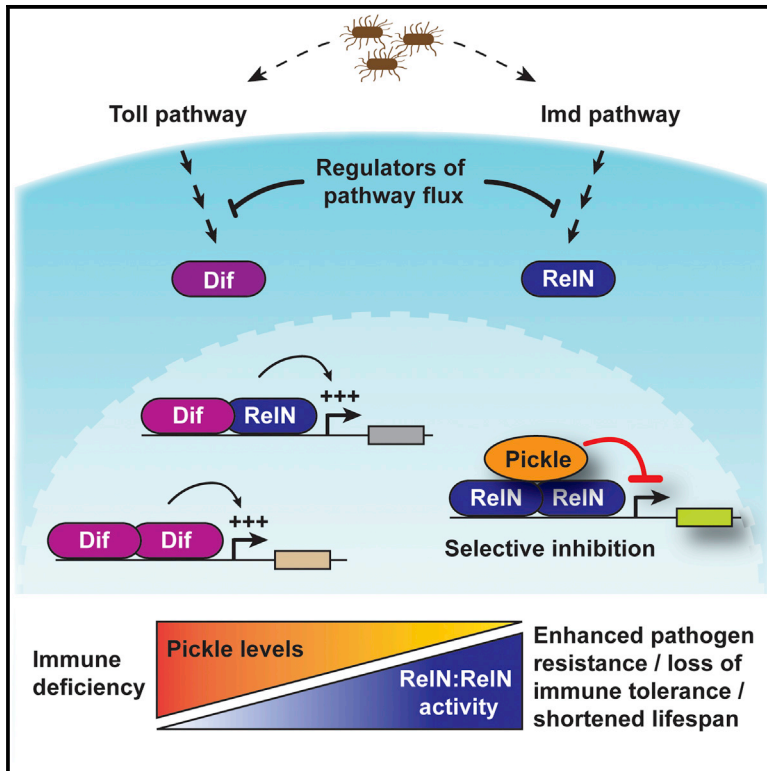


Cell Host & Microbe

Signal Integration by the I κ B Protein Pickle Shapes *Drosophila* Innate Host Defense

Graphical Abstract



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In Brief

Tight regulation of NF- κ B signaling is critical to avoid detrimental and misbalanced responses. Morris et al. identify an I κ B protein in *Drosophila* that inhibits a selective subset of the NF- κ B dimer repertoire, thereby ensuring an appropriate immune response to pathogens while preventing tissue damage and reduced lifespan.

Highlights

- Pickle selectively inhibits NF- κ B target genes that are driven by Relish homodimers
- Pickle is a nuclear member of the I κ B protein family
- Loss of *pickle* causes hyper-activation of Relish-dependent target genes
- Loss of *pickle* enhances host resistance to bacteria but compromises lifespan



Signal Integration by the I κ B Protein Pickle Shapes *Drosophila* Innate Host Defense

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<http://dx.doi.org/10.1016/j.chom.2016.08.003>

SUMMARY

Pattern recognition receptors are activated following infection and trigger transcriptional programs important for host defense. Tight regulation of NF- κ B activation is critical to avoid detrimental and misbalanced responses. We describe Pickle, a *Drosophila* nuclear I κ B that integrates signaling inputs from both the Imd and Toll pathways by skewing the transcriptional output of the NF- κ B dimer repertoire. Pickle interacts with the NF- κ B protein Relish and the histone deacetylase dHDAC1, selectively repressing Relish homodimers while leaving other NF- κ B dimer combinations unscathed. Pickle's ability to selectively inhibit Relish homodimer activity contributes to proper host immunity and organismal health. Although loss of *pickle* results in hyper-induction of Relish target genes and improved host resistance to pathogenic bacteria in the short term, chronic inactivation of *pickle* causes loss of immune tolerance and shortened lifespan. Pickle therefore allows balanced immune responses that protect from pathogenic microbes while permitting the establishment of beneficial commensal host-microbe relationships.

INTRODUCTION

Host defense against pathogen invasion relies on potent inflammatory responses that are controlled by the NF- κ B family of transcription factors (Hayden and Ghosh, 2008). Activation of these transcription factors sets in motion a program aimed at clearing the pathogen. To restore homeostasis of the infected organ, such programs also induce modulators that, through negative feedback, regulate their temporal outputs to achieve balanced immune responses upon infection (Pasparakis, 2009).

NF- κ B proteins share the presence of an N-terminal Rel homology domain (RHD), which is responsible for DNA binding as well as homo- and heterodimerization (Hayden and Ghosh, 2008). NF- κ B proteins carry either an extended C-terminal stretch that contains multiple copies of ankyrin repeats (p105, p100, and *Drosophila* Relish) or a C-terminal transcription activation domain (c-Rel, RelB, RelA [p65], and the *Drosophila* Dorsal [dl] and Dif [Dorsal-related immune factor] protein) (Gilmore, 2006). NF- κ B dimers bind to κ B sites within the promoters and enhancers of target genes and regulate transcription through the recruitment of coactivators and corepressors (Hayden and Ghosh, 2008). The combinatorial diversity of NF- κ B homo- and heterodimers contributes to the regulation of distinct, but overlapping, transcriptional programs (Smale, 2012).

The activity of NF- κ B is regulated by interaction with inhibitory I κ B proteins (Gilmore, 2006). The I κ B family proteins include, at least, eight dedicated I κ B proteins: I κ B α , I κ B β , I κ B γ , I κ B ϵ , I κ B ζ , I κ BNS, Bcl-3, and *Drosophila* Cactus. All I κ B proteins harbor multiple ankyrin repeat regions (ARRs) through which I κ Bs bind to the RHDs of NF- κ B dimers and regulate their transcriptional response. Generally, individual I κ Bs associate preferentially with a particular set of NF- κ B dimers (Gilmore, 2006). Studying the function, mechanism of activation, and regulation of these factors is crucial for understanding host responses to microbial infections, immunological memory, and commensal-host interactions.

Drosophila can engage two pathways to activate NF- κ B: the Toll pathway is activated primarily by fungal and Gram-positive infections, while the *Immune deficiency* (*Imd*) pathway responds mainly to Gram-negative infections (Buchon et al., 2014; Lemaitre et al., 1995, 1996).

Toll activation is triggered by Lys-type peptidoglycans (PGNs) as well as certain bacterial virulence factors and components of fungal cell walls (El Chamy et al., 2008; Gottar et al., 2006; Michel et al., 2001). The Toll pathway initiates via an extracellular proteolytic cascade that culminates in the cleavage and activation of Spatz (Spz), which binds to the transmembrane Toll receptor and initiates an intracellular signaling cascade that results in the phosphorylation-dependent degradation of the I κ B protein



Cactus (Ganesan et al., 2011). This enables nuclear translocation of the NF- κ B transcription factors Dif and dl (Lemaitre et al., 1996; Manfrulli et al., 1999; Rutschmann et al., 2002). Of these NF- κ B proteins, Dif is the predominant transactivator in the antifungal and anti-Gram-positive bacterial defense in adults (Lemaitre et al., 1996; Manfrulli et al., 1999; Meng et al., 1999; Rutschmann et al., 2000a). Dorsal can substitute for Dif in larvae (Manfrulli et al., 1999; Rutschmann et al., 2000b).

The Imd pathway is activated by Gram-negative bacteria via two DAP-type PGN recognition receptors, plasma-membrane PGRP-LC and cytosolic PGRP-LE (Buchon et al., 2014). Binding of PGN to the receptors results in recruitment of an Ub-dependent signaling complex consisting of Imd, dFadd, and the caspase-8 homolog Dredd (Ganesan et al., 2011). Dredd is activated in an Ub-dependent manner with the help of the E3-ligase inhibitor of apoptosis 2 (Diap2) (Kleino et al., 2005; Leulier et al., 2006; Meinander et al., 2012). Once active, Dredd cleaves off an inhibitory C-terminal ankyrin repeat of Relish, allowing translocation of the active RHD-containing N-terminal portion (RelN) to the nucleus, where it can act to induce activation of Relish-dependent target genes (Ganesan et al., 2011).

Activation of Toll and Imd pathways induces the expression of distinct but overlapping groups of NF- κ B responsive antimicrobial peptide (AMP) genes, which are important for fending off invading microorganisms (Buchon et al., 2014). Because dl, Dif, and RelN readily form homo- as well as heterodimers, the transcriptional output of NF- κ B can vary depending on dimer compositions and co-factor association (Bonnay et al., 2014; Busse et al., 2007; Goto et al., 2008; Han and Ip, 1999; Tanji et al., 2007, 2010). How organisms are able to detect the presence of pathogens, and in response trigger balanced expression of innate defense genes, is a major question. It is clear that the expression repertoire and duration of immune defense genes must be tightly balanced to effectively clear pathogens while avoiding deleterious immune activation and tissue damage. Whereas pathogens frequently trigger multiple pattern recognition receptors, it remains unclear how these signals are integrated into an appropriate defense response to clear the pathogen. Here we report the identification and characterization of a *Drosophila* member of the I κ B superfamily, which we term Pickle.

RESULTS

Pickle Negatively Regulates the NF- κ B Transcription Factor Relish

To identify regulators of NF- κ B signaling, we performed an in vitro RNAi mini-screen of proteins that interact with the *Drosophila* NF- κ B protein Relish (Guruharsha et al., 2011; Rhee et al., 2014). This identified CG5118 as a putative negative regulator of Relish (Figure 1). In S2⁺ cells, knockdown of CG5118, subsequently referred to as Pickle, caused hyperinduction of Imd-dependent AMP (AMP) genes following treatment with PGN from Gram-negative bacteria (Figures 1A, S1A, and S1B). Conversely, overexpression of Pickle strongly suppressed PGRP-LCx-, Imd-, and RelN-mediated induction of AMPs (Figures 1B–1D). This suggests that Pickle regulates the Imd pathway at the level of RelN. Accordingly, Pickle had no effect on Relish processing upon immune activation (Figure S1C).

Whereas Pickle inhibited both Imd- and RelN-mediated production of AMPs, Pirk suppressed only PGRP-LCx- and Imd-induced activation of AMP genes.

The observation that Pickle suppresses RelN-driven induction of AMPs strongly suggests that Pickle directly regulates active, processed Relish. Consistently, we found that Pickle readily bound to the RelN portion of Relish (Figures 1E, 1F, and S1D), which is in agreement with previous proteomic-based studies (Guruharsha et al., 2011; Rhee et al., 2014). Detailed interaction analysis revealed that Pickle homo-oligomerizes (Figure S1E) and that the C-terminal half (aa 277–525) of Pickle was necessary and sufficient for RelN binding (Figure 1F). Although Pickle efficiently bound to Relish, it did not interact with other members of the *Drosophila* NF- κ B family, such as dl and Dif (Figure S1D). Subcellular fractionation revealed that FLAG-tagged Pickle predominantly resides in the nuclear fraction (Figure 1G). Intriguingly, expression of Pickle appeared to sequester RelN in the nucleus, as significantly less RelN was present in the cytoplasmic fraction following co-expression with Pickle (Figure 1G).

The histone deacetylase dHDAC1 (also referred to as Rpd3) reportedly negatively regulates the transactivation of Relish (Kim et al., 2005, 2007), even though dHDAC1 does not directly bind to Relish (Kim et al., 2007). We therefore tested whether Pickle interacts with dHDAC1. We found that Pickle selectively co-purified endogenous dHDAC1 from cellular extracts (Figure 1H). Together, our data suggest that Pickle is a negative regulator of the Imd pathway that binds and inhibits the activity of the Relish, possibly via dHDAC1 recruitment.

Pickle Is a Member of the I κ B Superfamily of Proteins

All currently known I κ B proteins from vertebrates and invertebrates carry C-terminal ARR with which they bind to the RHDs of NF- κ B proteins (Hayden and Ghosh, 2008). Using sequence analysis and structural prediction algorithms, we identified seven ARRs within the C-terminal portion of Pickle (Figures 2A and S2), the portion that is necessary and sufficient for Relish binding. The N-terminal portion of Pickle did not harbor any recognizable motifs or domains. Because Pickle selectively binds to the RHD of Relish via its C-terminal ARRs and inhibits Relish activity, Pickle fulfills all functional and structural criteria of I κ B proteins.

Phylogenetic analysis of Pickle with all currently known I κ Bs revealed that Pickle, along with its orthologs, is part of a clade of the I κ B phylogenetic tree. I κ B phylogenetic rooted tree reconstruction identified five major clades among the I κ B proteins (Figure 2B). These major clades include (1) Pickle and Relish with NF- κ B1 and NF- κ B2 (53.4% bootstrap value), (2) Cactus with I κ B α (61.3% bootstrap value), (3) I κ B ϵ (95.4% bootstrap value), (4) I κ B β (99.7% bootstrap value), and (5) Bcl-3 with I κ B ζ and I κ BNS (nuclear I κ B proteins; 50.9% bootstrap value). The tree organization was validated using rooted and unrooted phylogenetic trees of invertebrate I κ Bs (Figure 2C). Pickle clustered along with Relish in both whole I κ B and invertebrate-specific phylogenetic trees, with a bootstrap support of 100%. Our distance analysis demonstrates that *pickle* represents the direct arthropod homolog of the *relish* gene, albeit lacking a RHD in the N terminus and a PEST domain in its C terminus. Taken together, our functional, phylogenetic, and sequence analysis identifies Pickle, and its orthologs, as a member of the I κ B superfamily.

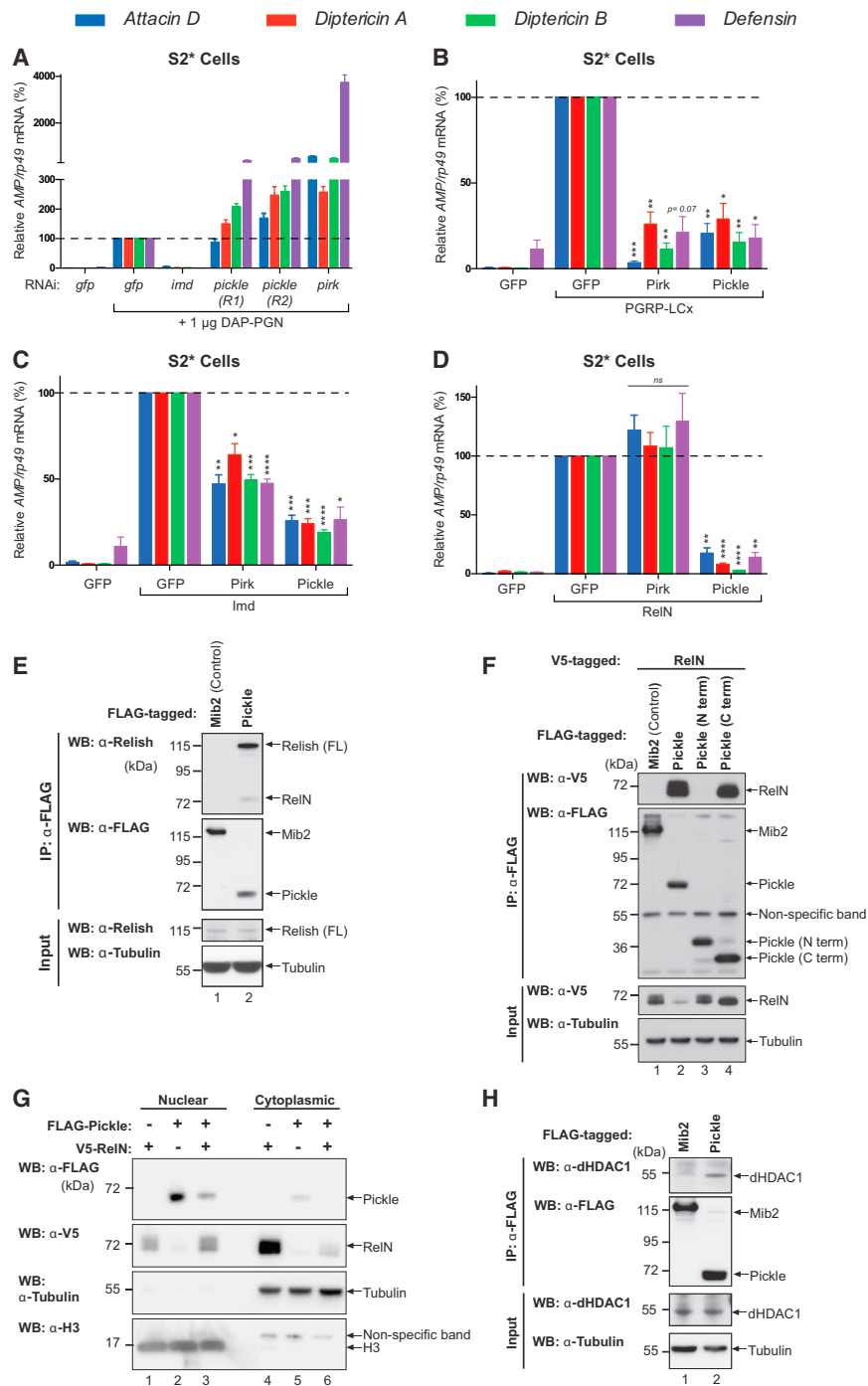


Figure 1. Pickle Negatively Regulates Relish

(A–D) qRT-PCR analysis of mRNA from S2* cells. (A) Relative AMP mRNA levels before and after 4 hr of treatment with DAP-PGN in the presence of the indicated double-stranded RNAs (dsRNAs). R1 and R2 depict dsRNAs targeting two non-overlapping regions (R) of *pick*. (B–D) Relative AMP mRNA levels of S2* cells transiently transfected with the indicated constructs. V5-tagged RelN was used.

(E and F) FLAG immunoprecipitation of the indicated proteins was performed in S2* cells, and Relish binding was assessed via western blot.

(G) Nuclear and cytoplasmic extracts of S2* cells transfected with the indicated proteins were analyzed by western blot. Equal total protein was loaded for both extracts.

(H) FLAG-tagged Pickle and FLAG-tagged Mib2 (control) were expressed in S2* cells. FLAG immunoprecipitation was performed and binding of endogenous dHDAC1 to Pickle, or Mib2, was assessed via western blot.

Histograms express results as percentage of a control sample (marked with dotted line). Unless otherwise indicated, p values were calculated from control using an unpaired Student's t test. Results are representative of three (B–H) or two (A) biological repeats. Mean ± SEM of biological (B–D) or experimental (A) repeats. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001. See also Figure S1.

of *Drosomycin* following activation of the Toll pathway via septic injury with the Gram-positive, Lys-type PGN containing bacteria *Micrococcus luteus* (*M.lut*) (Figures S3C and S3D). Pickle, therefore, selectively modulates Imd signaling.

pick also controlled the Imd pathway in the fly midgut following oral infection. Accordingly, feeding Gram-negative *Ecc15* or *Pseudomonas entomophila* (*P.e*) caused upregulation of multiple Relish target genes in dissected midguts (Figures 3B and S3E). Compared with control flies, induction of Relish-dependent genes was significantly greater in flies with enterocyte-specific knockdown of *pick* (Figures 3B, S3A, S3B, and S3E). *pick*^{P1EPgy2|EY18569} null mutant flies (hereafter referred to as *pick*^{ey}), which carry a

Loss of *pick* Results in Hyper-Activation of Relish Target Genes upon Infection

Next we investigated the role of Pickle in regulating *Drosophila* innate immune responses. Septic injury with the Gram-negative bacteria *Erwinia carotovora carotovora* 15 (*Ecc15*) resulted in hyper-activation of Imd signaling in flies in which *pick* was knocked down in the fat body (Figures 3A and S3A). Although knockdown of *pick* resulted in hyper-activation of Relish target genes, *pick* inactivation did not affect Dif-mediated induction

transposon inserted 24 bp downstream of the translational start site of *pick* (Figures 3C and S3G), also hyper-activated Relish target genes following systemic infection with *Ecc15* (Figure 3D). Likewise, oral infection with *Ecc15* or *P.e* similarly caused a dramatic over-production of Relish-dependent target genes (Figures 3E and 3G). Essentially the same results were obtained using either homozygous *pick*^{ey} mutant animals or trans-heterozygous *pick*^{ey/Df1} or *pick*^{ey/Df2} flies that carry deletions of the *pick* locus (*Df1: Df[2L]Exel7006; Df2: Df[2L]BSC481*) (Figures

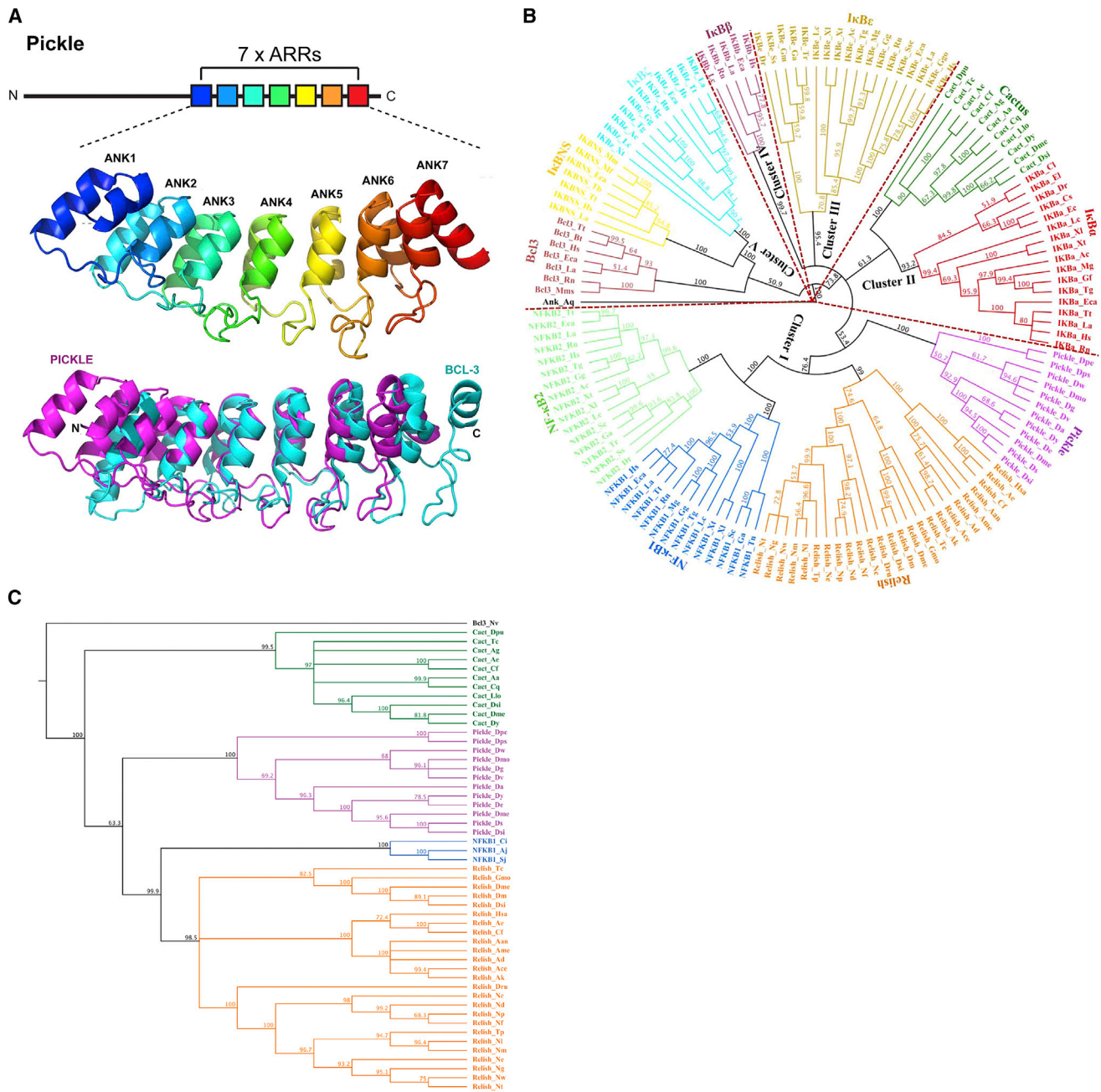


Figure 2. Phylogenetic Relationship of Pickle with Other I κ B Family Members

(A) Schematic representation of Pickle (top) and its predicted 3D structure (middle). The predicted structure of the seven ARRs of Pickle (magenta) was superimposed onto the structure of Bcl-3 (PDB: 1K1A; cyan) (bottom).

(B) Phylogenetic analysis of I κ B proteins. The sponge *Amphimedon queenslandica* was considered as an out-group. Bootstrap values > 50% have been provided. Members: I κ B α (red), I κ B β (wine), I κ B ϵ (tan), Bcl-3 (brown), I κ BNS (yellow), I κ B ζ (cyan), Cactus (dark green), Relish (orange), NF- κ B1 (blue), NF- κ B2 (light green), and Pickle (magenta).

(C) Phylogenetic relationship of Pickle with I κ B family members present in invertebrates only using neighbor-joining method. Bcl-3 from *Nematostella vectensis* was considered as an outgroup (shown in black). Bootstrap scores > 60% have been provided. Members: Cactus (dark green), Relish (orange), NF- κ B1 (blue), and Pickle (magenta).

See Table S1 for details. See also Figure S2.

3D, 3E, 3G, and S3F). Of note, following systemic infection, *Defensin* induction was strongly reduced in homozygous *pickle*^{ey} flies when compared to wild-type (WT) animals (*yw* and *w*¹¹¹⁸). This ef-

fect is due to a background mutation in *pickle*^{ey} flies because the reduced *Defensin* levels did not complement when *pickle*^{ey} was placed trans-heterozygous over *pickle*-uncovering deficiency

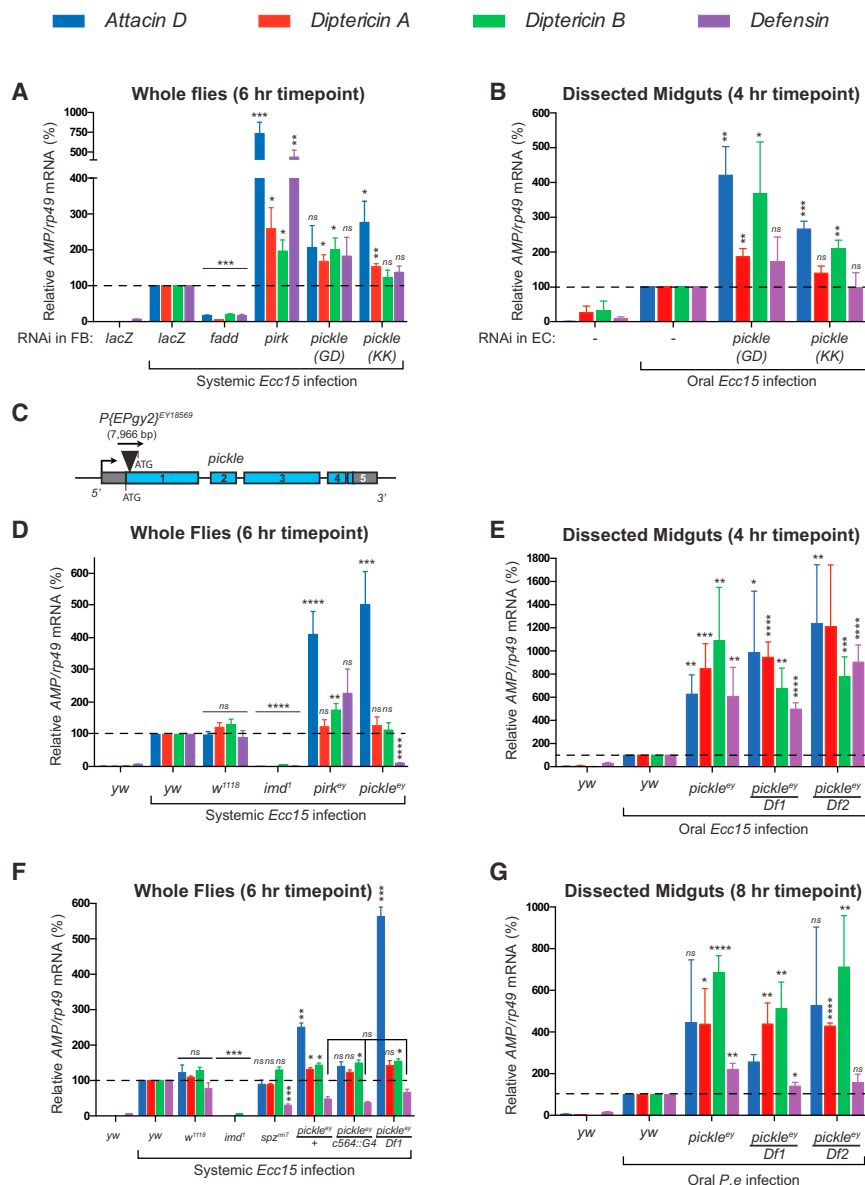


Figure 3. Loss of *pick* Causes Hyperinduction of AMPs following Infection with Gram-Negative Bacteria

(A, B, and D–G) qRT-PCR analysis of the indicated genotypes. (A) Relative AMP mRNA levels from whole flies before and after 6 hr of infection with *Ecc15* (~2,000 CFU). RNAi of the indicated target genes was driven in the fat body (FB) using *c564::Gal4*. *pick* (GD) and *pick* (KK) refer to two transgenic lines encoding dsRNAs that target non-overlapping regions of *pick*. (B) Relative AMP mRNA levels of dissected midguts before and after 4 hr of oral infection with *Ecc15*. RNAi knockdown was restricted to enterocytes (EC) using *myo::Gal4*.

(C) Schematic representation of the *pick* gene depicting the insertion site of the transposon P[EPgy2]^{EY18569}.

(D and E) The indicated flies were treated as in (A) and (B), respectively. *Df1* refers to the *Df(2L) Exel7006* deletion. *Df2* refers to *Df(2L)BSC481*.

(F) The indicated flies were analyzed as in (A). (G) Relative AMP mRNA levels from dissected midguts before and after 8 hr of infection with *P.e* oral infection.

Histograms express results as percentage of a control sample (marked with dotted line). Unless otherwise indicated, p values were calculated from control using an unpaired Student's t test. Results are representative of at least three biological repeats (mean ± SEM). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001. See also Figure S3.

alleles (Figures 3F and S3F). This background effect only affects the expression of *Defensin*, not other AMPs, and was observed following only systemic, not oral, infection (Figures 3D–3G and S3F). This is evident as oral infection with *Ecc15* or *P.e* caused elevated *Defensin* levels in homozygous *pick^{ey}* flies that were comparable with those of *pick^{ey}/Df1* and *pick^{ey}/Df2*. To circumvent this background effect, all subsequent systemic infection experiments were conducted using *pick^{ey}/Df1*, *pick^{ey}/+*, and *pick^{ey}/c564* genotypes, allowing the comparison of flies with zero (*pick^{ey}/Df1*) or one WT copy (*pick^{ey}/+*) and one WT copy with one allele re-expressing Pickle in the fat body (*pick^{ey}/c564*). Together, our data indicate that *pick* negatively regulates the Imd pathway, upon both systemic and oral infections.

Although loss of *pick* resulted in hyper-activation of Relish target genes following systemic infection, fat body-specific and P[EPgy2] transposon-mediated re-expression of *pick* fully rescued AMP expression to normal levels (Figure 3F).

in *pick^{ey}* flies, our data indicate that the *pick^{ey}* phenotype is indeed due to loss of *pick*.

***pick* Suppresses Spontaneous Induction of Relish-Dependent Target Genes in the Absence of Infection and Maintains Fly Lifespan**

For a host to tolerate a certain amount of resident bacteria, it is critical that the activation threshold of the immune response be tightly regulated (Buchon et al., 2014). Because *pick* is a selective negative regulator of Relish, we investigated whether *pick* contributes to the activation threshold of Relish-dependent target genes by suppressing Relish activity. Using the sterile environment of S2⁺ cells, we found that mere knockdown of *pick* led to a dramatic induction (>5,000-fold) of the basal levels of *Diptericin A* (*DiptA*) and *Diptericin B* (*DiptB*) (Figure 4A). Likewise, tissue-specific knockdown of *pick* in the gut (enterocytes) or fat body led to a marked increase in the basal levels

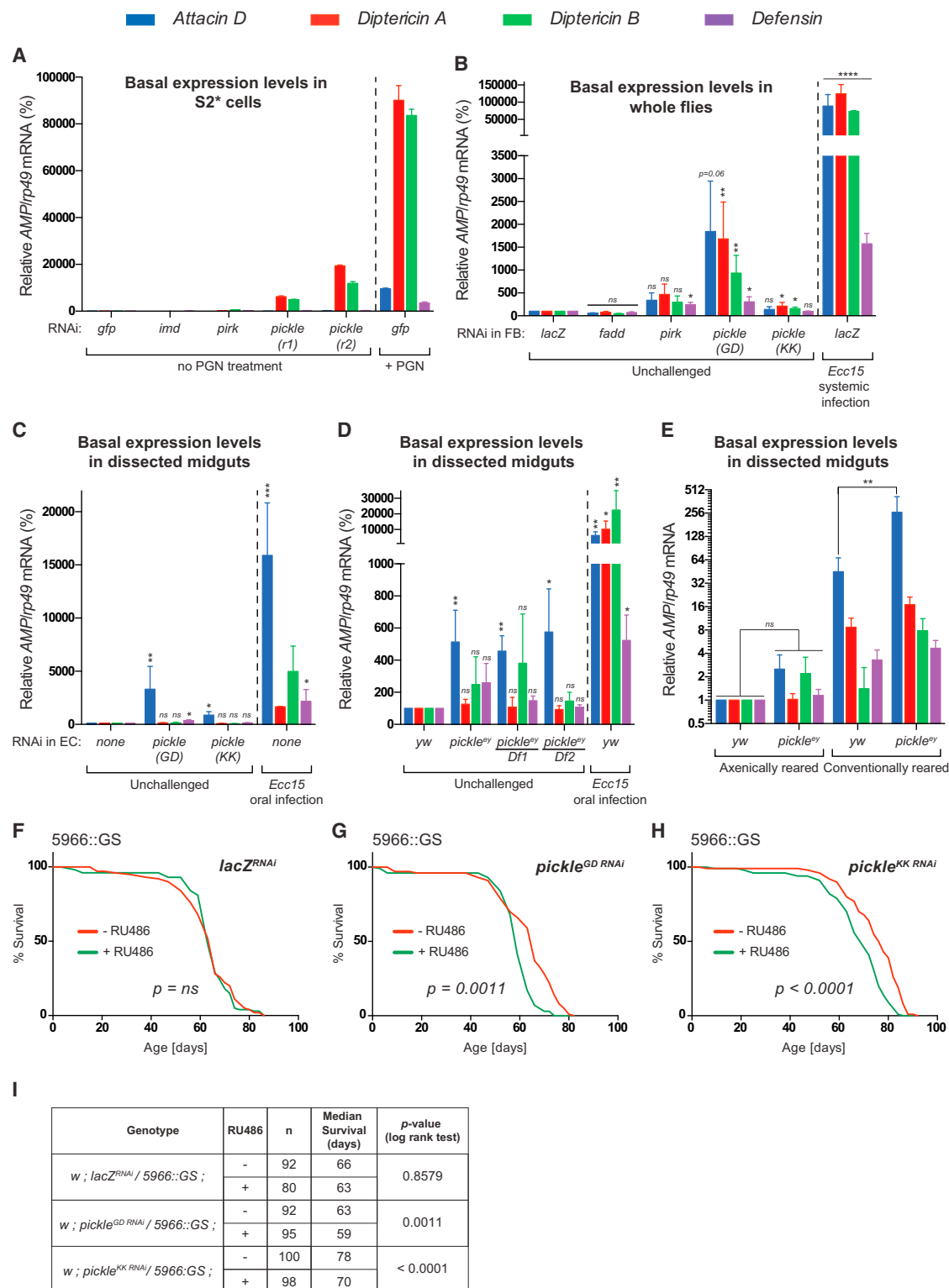


Figure 4. *pickle* Suppresses Spontaneous Induction of Relish-Dependent Target Genes in the Absence of Infection and Maintains Lifespan (A–E) qRT-PCR analysis of mRNAs of the indicated samples. (A) Relative AMP mRNA levels from unchallenged S2* cells following RNAi of the indicated genes. S2* cells treated for 4 hr with DAP-PGN are shown as reference point. (B) Relative AMP mRNA levels from unchallenged whole flies. RNAi knockdown was restricted to fat body (FB) cells using *c564::Gal4*. Relative AMP mRNA levels of control flies injected with *Ecc15* (2,000 CFU) (6 hr) served as reference point. (C) Relative AMP mRNA levels of dissected midguts from unchallenged female flies. RNAi knockdown was restricted to enterocytes (EC) using *myo::Gal4*.

(legend continued on next page)

of *AMP* gene expression in unchallenged flies (Figures 4B and 4C). Transcript levels of *AMP* genes were also significantly elevated in dissected midguts of unchallenged *pickle^{ey}* and trans-heterozygous *pickle^{ey/Df1}* and *pickle^{ey/Df2}* animals (Figure 4D). However, unlike in *S2** cells, the elevated expression of *AMPs* in midguts of *pickle^{ey}* flies was dependent on the presence of commensal bacteria, as this phenotype was lost when flies were reared under sterile conditions (Figure 4E). These data suggest that Pickle contributes to immune tolerance in the gut, preventing aberrant Relish-activity in response to gut microbiota. The difference between *S2** cells and cells of the midgut may reflect cell- and tissue-type dependent differences.

Previous work indicated that chronic hyper-activation of Imd signaling in the gut reduces lifespan (Guo et al., 2014; Paredes et al., 2011). To test whether loss of *pickle* impacts on lifespan, we made use of the GeneSwitch system (Mathur et al., 2010), which negates genetic background effects (He and Jasper, 2014). Consistent with the notion that gut-specific knockdown of *pickle* results in hyper-activation of Imd signaling, we found that long-term, GeneSwitch-mediated depletion of *pickle* in enteroblasts and enterocytes caused a significant reduction in lifespan (Figures 4G–4I). Under the same conditions, GeneSwitch-mediated depletion of *lacZ* had no effect (Figures 4F and 4I). Together these data demonstrate that depletion of *pickle* results in hyper-activation of Imd signaling in the gut, which, similar to the loss of other Imd pathway negative regulators (Paredes et al., 2011), may compromise lifespan.

***pickle* Is Induced in Response to Commensal and Infectious Bacteria**

Expression of several negative regulators of the Imd pathway, such as *pirk* and *PGRP-LB*, are regulated by Relish, allowing negative-feedback control of Imd signaling (Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008; Zaidman-Rémy et al., 2006). We found that *pickle* levels were significantly higher in midguts of conventionally reared (CR) animals than in germ free (GF) counterparts (Figure 5A). This indicates that *pickle* expression in the midgut is influenced by the presence of commensal bacteria, an observation that is consistent with a recent micro-array study (Broderick et al., 2014). Following oral infection, induction of *pickle* varied depending on the type of Gram-negative bacteria. Whereas oral infection with *Ecc15* did not induce *pickle* expression (Figure 5B), exposure to the entomopathogenic bacteria *P.e* caused a significant increase in *pickle* expression (Figure 5C). A similar bacteria-specific induction of *pickle* was also noted previously (Buchon et al., 2009a; Chakrabarti et al., 2012). Unlike *pickle*, expression of *pirk* increased in response to both these Gram-negative bacteria (Figures 5B–5D). Consistent with the notion that *pickle* and *pirk* are regulated differently, we found that exposure to *P.e* induced

pickle independently of *PGRP-LC/LE*, *Imd*, and *Relish* (Figure 5D). Upon systemic infection, the induction of *pickle* is relatively modestly (<2 times) (Figure S4A), which is in agreement with previous micro-array studies (De Gregorio et al., 2002; Irving et al., 2001). This was unlike *pirk*, which was strongly upregulated in an Imd-dependent manner upon systemic infection (Figure S4B). Although the pathway or pathways that regulate *pickle* expression remain to be identified, *pickle* expression in the midgut appeared not to be induced by tissue damage per se (Figures S4C and S4D). Together, our data demonstrate that *pickle* is induced, albeit moderately, in response to commensal microbiota, and infection with certain types of bacteria.

Pickle Selectively Inhibits RelN Homodimers

The RHD of NF- κ B proteins mediates DNA binding as well as homo- and heterodimerization (Hayden and Ghosh, 2008). In *Drosophila*, concomitant activation of the Toll and Imd pathways reportedly drives the formation of a complex network of Dif, dl, and Relish homo- and heterodimers (Tanji et al., 2010). Different dimer combinations are thought to activate overlapping transcriptional programs that vary in intensity, duration, and target genes (Smale, 2012). Because Pickle selectively binds to RelN (Figure 1), we tested the ability of Pickle to regulate various NF- κ B homo- and heterodimer combinations. Whereas expression of Pickle strongly suppressed the transactivation ability of RelN as well as linked RelN[^]RelN homodimers (Figures 6A, 6F, S5A, and S5F; the caret represents the flexible peptide linker), Pickle failed to inhibit Dif, dl, and linked dl[^]RelN or Dif[^]RelN dimer combinations (Figures 6B–6E and S5B–S5E). Of note, the ability of Pickle to repress induction of *AttD* and *AttA* was irrespective of the level of induction (Figures 1D and S5G–S5J). Intriguingly, the inability of Pickle to suppress linked Dif[^]RelN and dl[^]RelN was not due to lack of Pickle-binding, as Pickle readily co-purified Dif[^]RelN and dl[^]RelN from cellular extracts (Figure S5K). This suggests that Pickle requires two RelN moieties to inhibit transactivation.

Next, we investigated the impact of Pickle when both the Imd and Toll pathways are simultaneously activated in vivo. To that end, we used injection of heat-killed (hk) *E. coli* (*E.coli*) and *M.lut* and examined gene expression after 6 hr. Heat-killed bacteria were used to avoid any complication due to different bacterial growth rates. Interestingly, we found that loss of *pickle* (*pickle^{ey}* and *pickle^{ey/Df1}*) hyper-activated *AttD* only when *AttD* was driven by RelN-only, such as following injection with *E.coli* (hk) (Figures 6G–6J, S5L, and S5M). In contrast, loss of *pickle* had no effect on *AttD* expression following co-injection of *E.coli* (hk) + *M.lut* (hk) (Figure 6H), a condition that induces *AttD* expression in an Imd- and Toll-dependent manner. This is entirely consistent with the notion that Pickle selectively inhibits target gene induction when such genes are exclusively

(D) Analysis of flies with the indicated genotypes was conducted as in (C). (E) Relative *AMP* mRNA levels of dissected midguts from unchallenged female flies reared under conventional or axenic conditions.

(F–H) Lifespan experiments using the geneswitch system. Knockdown was restricted to enteroblasts (EBs)/enterocytes (ECs) using the geneswitch driver 5966:GS.

(I) Statistical summary of experiments shown in (F–H).

Histograms express results as percentage of a control sample (marked with dotted line). Unless otherwise indicated, p values were calculated from control using an unpaired Student's t test. Results are representative of three (B–E) or two biological repeats (A). Mean \pm SEM of biological (B–E) or experimental (A) repetitions.

*p \leq 0.05, **p \leq 0.01, ***p \leq 0.001, and ****p \leq 0.0001.

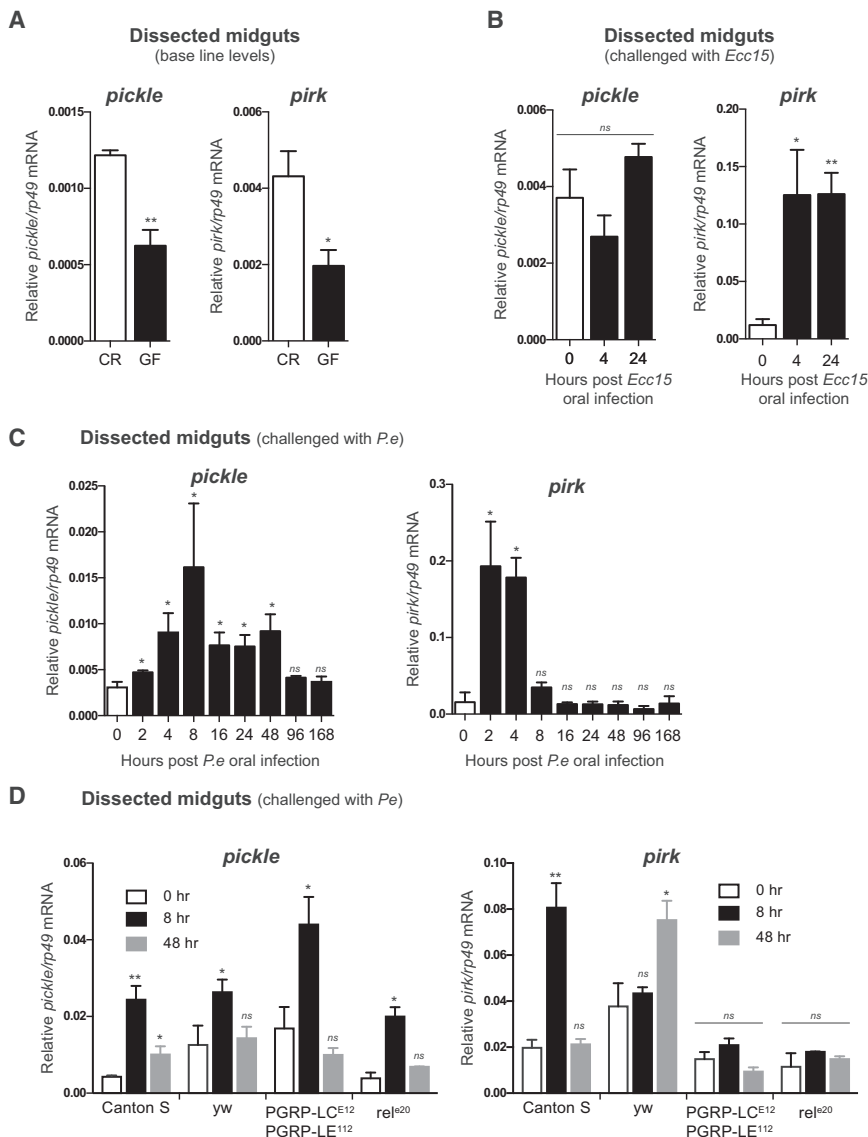


Figure 5. *pickle* Expression Is Induced in Response to Commensal and Infectious Bacteria

(A–D) qRT-PCR analysis of *pickle* or *pirk* transcript levels. Unless otherwise stated, results are from dissected midguts of Canton S flies. (A) Relative mRNA levels of *pickle* and *pirk* in CR and GF flies. (B) Relative mRNA levels of *pickle* and *pirk* following oral infection with *Ecc15* or (C) *P.e*. (D) Relative mRNA levels of *pickle* and *pirk* in dissected midguts of the indicated genotypes following oral infection with *P.e*. p values were calculated from respective control (white bars) using an unpaired Student's t test. Results are representative of three biological repetitions (mean \pm SEM). *p \leq 0.05, **p \leq 0.01, ***p \leq 0.001, and ****p \leq 0.0001. See also Figure S4.

***pickle* Alters Host Resistance following Infection with Pathogenic Bacteria**

To study the physiological relevance of *Pickle* in selectively inhibiting RelN homodimers, we examined the response of *pickle* mutants to infection with the pathogenic bacteria *L. monocytogenes* (*L.mono*) and *P. rettgeri* (*P.ret*). Six hours after infection, both these bacteria activated also the Toll pathway in addition to the Imd pathway (Figures 7A, 7B, S6A, and S6B) (Buchon et al., 2009b; Gordon et al., 2005). Although these bacteria activated both the Imd and Toll pathways, some AMPs (*Defensin*) displayed different pathway dependency depending on the infecting bacteria. Induction of *AttD* in response to *L.mono* and *P.ret* infection was dependent solely on the Imd pathway (Figures 7A and 7B). *Defensin*, on the other hand, was solely Imd-dependent upon *L.mono* infection, whereas it was

driven by RelN. Of note, the overall level of *AttD* induction did not influence the ability of *pickle* to regulate RelN-driven expression of *AttD*. This is evident as injection of live *Ecc15*, which drives *AttD* induction in a purely Imd-dependent manner, triggered the strongest upregulation of *AttD* (Figure 6I). Nevertheless, loss of *pickle* caused significant hyper-activation of *AttD*. Overall, our data strongly suggest that *Pickle* selectively inhibits RelN homodimers, while leaving Dif:RelN heterodimers unscathed (Figure 6J). Of note, at present we cannot rule out the possibility that synergistic induction of AMPs is mediated by cooperating homodimers (Figure S5N), instead of heterodimers. Regardless of whether the *Drosophila* NF- κ B proteins can act as either self-contained heterodimers or cooperating homodimers, our data clearly demonstrate that *Pickle* only affects target gene expression when such genes are driven exclusively by RelN-only. As such, these data are entirely consistent with our observations using compound NF- κ B dimers in S2* cells.

co-dependent on the Imd and Toll pathways following infection with *P.ret*. Interestingly, loss of *pickle* hyper-activated *AttD* and *Defensin* only when these AMPs were driven solely by RelN, such as following infection with *L.mono* (*AttD* and *Defensin*) and *P.ret* (*AttD*). Likewise, *c564::Gal4*-driven re-expression of *pickle* rescued the levels of *AttD* and *Defensin* expression to WT levels only when these AMPs were exclusively driven by RelN (Figure 7A). In contrast, loss of *pirk* caused hyper-activation of *AttD* and *Defensin* irrespective of the infecting bacteria, and irrespective of whether these AMPs were driven in an Imd- or Imd/Toll-dependent manner. Unlike *AttD* and *Defensin*, expression of *DiptA* and *DiptB* was insensitive to modulation by negative regulators such as *pirk* or *pickle*, quite possibly because these AMPs are already maximally induced. Our data are consistent with the notion that *Pickle* affects NF- κ B target gene expression only when such genes are driven exclusively by RelN-only.

Next, we tested the ability of *pickle* to modulate the survival of flies infected with *L.mono*, *P.ret*, and *B. subtilis* (*B.sub*). *B.sub* is

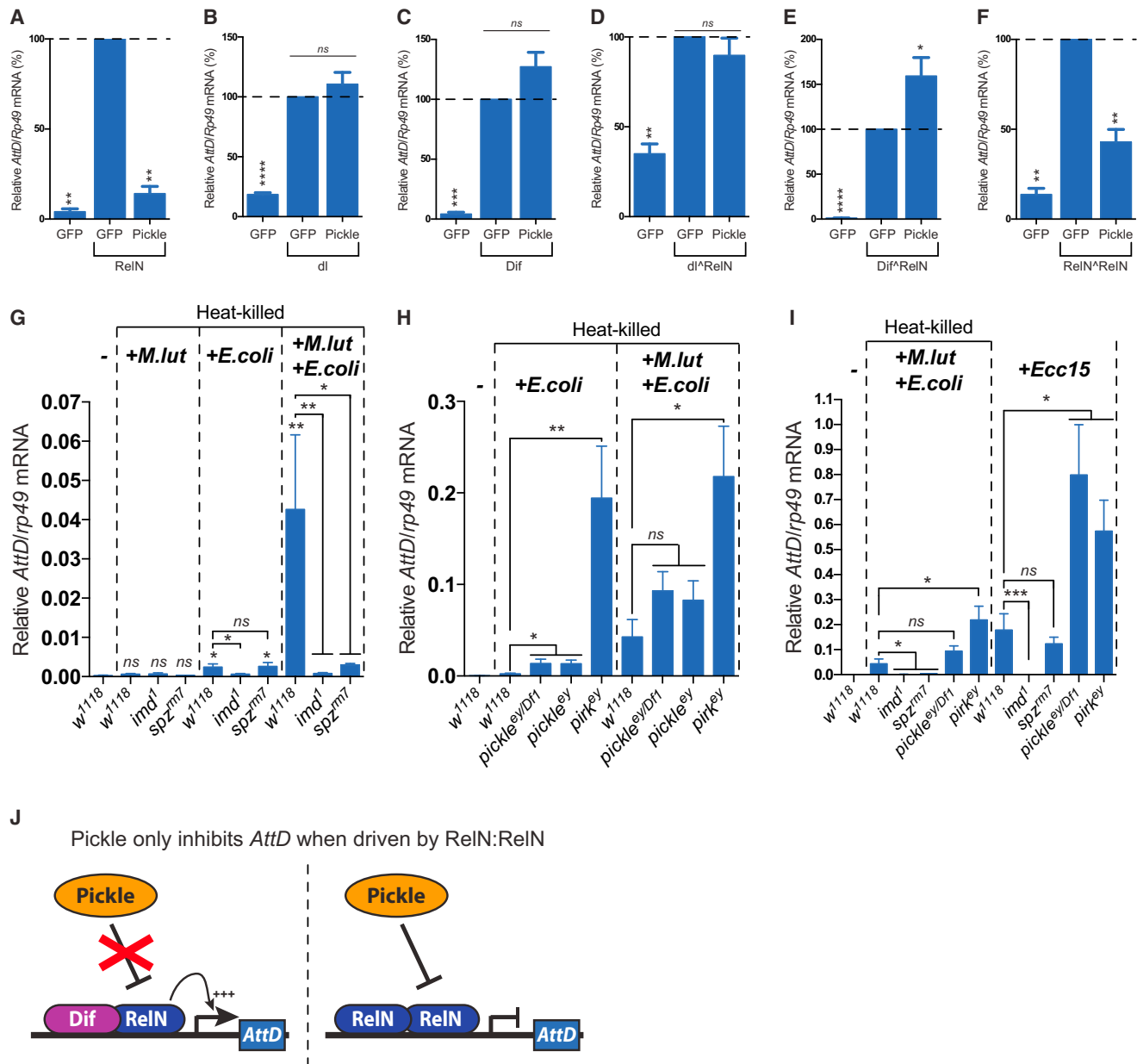


Figure 6. Pickle Selectively Inhibits RelN Hmodimers

(A–F) Relative *AttD* mRNA levels of S2 cells transiently transfected with plasmids expressing the indicated proteins. All proteins are FLAG-tagged at their N termini. Histograms depict mean \pm SEM of three biological repeats. Results are expressed as percentage of induced GFP control samples in each experiment, and statistical significance is measured from these using an unpaired Student's t test.

(G–I) Relative *AttD* levels mRNAs from unchallenged flies or flies injected with the indicated hk or live (*Ecc15*, 2,000 CFU) bacteria (6 hr). Unless otherwise indicated, statistical significance was measured from unchallenged *w¹¹¹⁸* flies using an unpaired Student's t test.

(J) Model depicting Pickle-mediated regulation of RelN.

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$. See Figure S5.

another pathogenic bacteria that activates both Imd and Toll pathways (Buchon et al., 2009b). Interestingly, *pick^{ey/Df1}* mutant flies were significantly less susceptible to systemic infection with *L.mono*, *P.ret*, and *B.sub* (Figures 7C, 7E, 7F, and S6C). In some instances, *pick^{ey}* appeared haploinsufficient, as *pick^{ey/+}* flies were significantly protected against *L.mono* and *P.ret* (~200 colony-forming units [CFU]) infection compared

with WT animals (*w¹¹¹⁸*). Notably, this was dependent on bacterial dose, as at a higher dose (~10,000 CFU), *pick^{ey/+}* and WT flies rapidly succumbed to *P.ret* infection, whereas *pick^{ey/Df1}* flies were significantly protected (Figure 7F). *c564::Gal4*-mediated re-expression of *pick^{ey}* in the fat body re-sensitized heterozygous flies to systemic bacterial infection (Figures 7C, 7E, and S6C), corroborating the specificity of the observed phenotype.

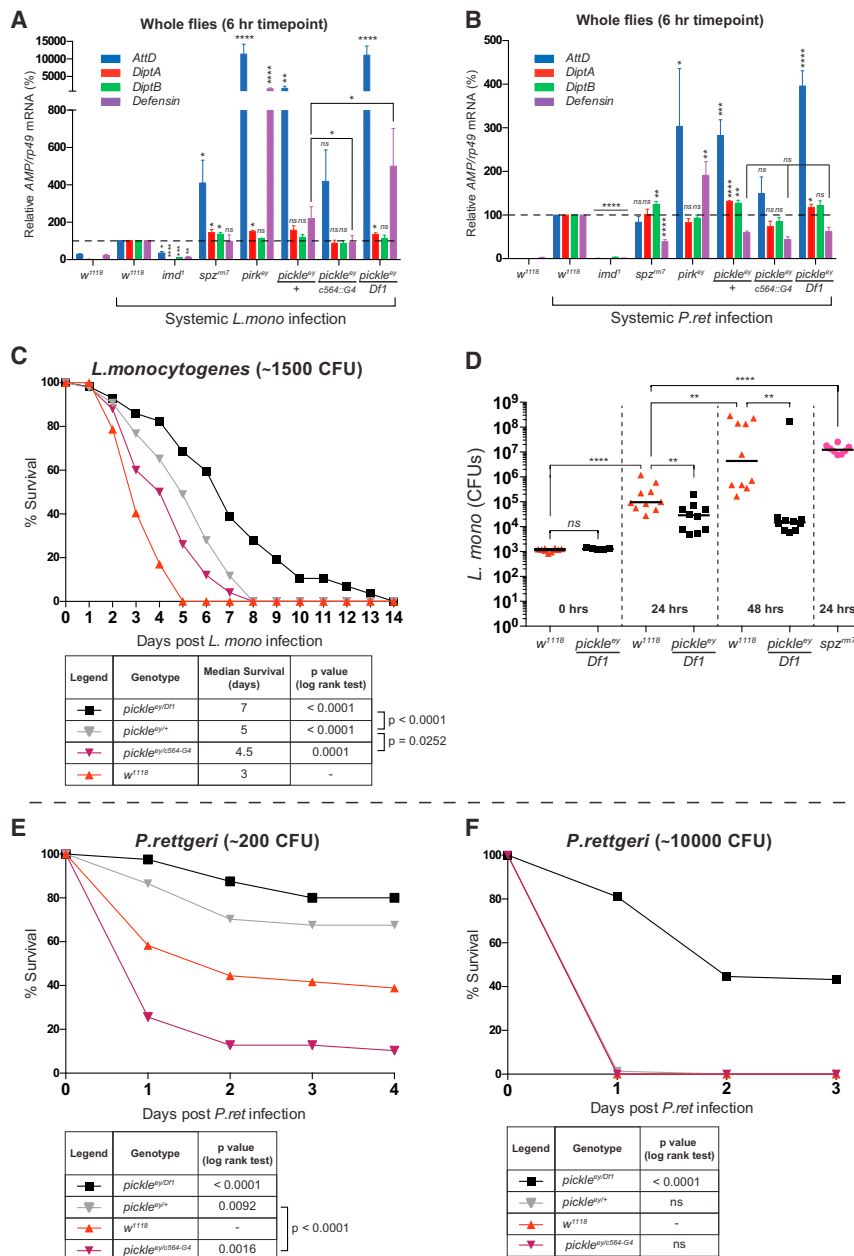


Figure 7. Loss of *pickle* Improves Host Resistance to Pathogenic Bacteria

(A and B) qRT-PCR analysis of AMP mRNAs of the indicated flies before and 6 hr post-systemic infection with (A) *L. mono* (~1,500 CFU) and (B) *P. ret* (~10,000 CFU). Results are expressed as percentage of the induced levels of control flies (*w¹¹¹⁸*) in each experiment (marked with a dotted line), and statistical significance was measured from these using an unpaired, two-tailed Student's *t* test. Histograms depict mean \pm SEM of three biological repetitions.

(C) Kaplan-Meier plot showing the survival of female flies injected with *L. mono* (~1,500 CFU). Statistical significance between the survival of infected flies and WT controls (*w¹¹¹⁸*) was determined using log rank tests; $n \geq 45$ flies for each genotype.

(D) Persistence of *L. mono* in *w¹¹¹⁸*, *pickle^{ey/Df1}*, and *spz^{tm7}* flies, measured at the indicated time points. All flies were injected with an identical initial dose of *L. mono* (~1,500 CFU). Statistical significance was determined using a Mann-Whitney U test.

(E and F) Kaplan-Meier plot showing the survival of female flies injected with (E) ~200 CFU or (F) ~10,000 CFU *P. ret*. Statistical significance between the survival of infected flies and a control *w¹¹¹⁸* strain was determined using log rank tests; $n \geq 45$ flies for each genotype.

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$. See also Figure S6.

kis, 2012). Here, we demonstrate that Pickle is required to prevent hyper-activation of Relish-dependent target genes. While loss of *pickle* improves host resistance to a variety of pathogenic bacteria, chronic inactivation of *pickle* compromises immune tolerance and shortens overall lifespan.

Pickle is a member of the I κ B superfamily of proteins that selectively suppresses the production of Relish-dependent target genes. Like other I κ B proteins, Pickle harbors C-terminal ARRs through which it binds to the RHD

The enhanced resistance of *pickle^{ey/Df1}* flies to *L. mono* was accompanied with a reduced bacterial load. Accordingly, *pickle^{ey/Df1}* flies harbored significantly fewer *L. mono* CFUs at 24 and 48 hr post-infection compared with WT controls (Figure 7D). Because *rel^{e20}* or *imd¹* mutant flies are acutely sensitive to infection with *B. sub*, *L. mono*, or *P. ret* (Figures S6D–S6F) (Buchon et al., 2009b; Mansfield et al., 2003), our data are consistent with a model whereby loss of *pickle* results in enhanced RelN-dependent immunity.

DISCUSSION

Tight regulation of NF- κ B signaling is critical, as misbalanced and prolonged responses are detrimental to the host (Paspara-

of Relish and inactivates Relish-mediated target gene expression, possibly via the recruitment of the histone deacetylase dHDAC1. Even though Pickle can bind to tethered Dif \wedge RelN and dI \wedge RelN heterodimers, it suppresses NF- κ B target gene expression only when such genes are driven solely by RelN. Accordingly, expression of Pickle strongly suppresses the trans-activation ability of RelN as well as RelN \wedge RelN homodimers (Figures 6). By contrast, Pickle fails to inhibit Dif, dI, and linked dI \wedge RelN or Dif \wedge RelN dimer combinations. Moreover, under conditions in which the Toll and Imd pathways are simultaneously activated, *pickle* exclusively influences induction of AMPs that are driven by RelN-only (Figures 6 and 7). Pickle, therefore, likely “skews” the output of both pathways via selective inhibition of genes solely transactivated by Relish. This is unlike Pirk, which

regulates pathway flux, and does not selectively inhibit a specific subset of the NF- κ B dimer repertoire.

Although homo- and heterodimers mediate diverse effects in mammalian systems (Hayden and Ghosh, 2008), it has been suggested that in *Drosophila* NF- κ B proteins might mediate their effects as cooperating homodimers bound to distinct κ B sites, rather than as heterodimers bound to a single site (Busse et al., 2007). Despite good evidence to suggest that heterodimers function in *Drosophila* (Han and Ip, 1999; Senger et al., 2004; Tanji et al., 2010), we cannot rule out the possibility that synergistic induction of AMPs is mediated by cooperating homodimers. Regardless of whether the *Drosophila* NF- κ B proteins can act as either self-contained heterodimers or cooperating homodimers, our data demonstrate that Pickle inhibits AMP induction only when RelN is the only NF- κ B member driving target gene expression. Under conditions in which AMPs are driven cooperatively by Dif and RelN, or Dif and dl, AMP production is insensitive to the presence of Pickle.

Pickle's ability to bias the output of certain Relish-dependent target genes, namely, those that are driven solely by RelN:RelN, has important physiological consequences. In the short term, loss of *pickle* enhances expression of RelN target genes, significantly boosting the host defense from infection with pathogenic bacteria. Although we observed elevated levels of several AMPs in *pickle* mutant flies, mere hyper-activation of these AMPs was not the only reason these animals were protected. *pirk* mutant animals similarly hyper-activated these AMP genes, yet these animals were unable to fend off *L.mono*, *P.ret*, and *B.sub*. The difference between loss of *pickle* and loss of *pirk* is likely due to the differential regulation of Imd signaling. Because Pirk regulates Imd signaling at the level of the receptor (or Imd) (Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008), Pirk is unable to skew the Imd and Toll signaling outputs toward a subset of NF- κ B target genes that are driven by a particular NF- κ B dimer combination. Although in the short term, loss of *pickle* appears to be beneficial for immune defense against certain pathogenic bacteria (*L.mono*, *P.ret*, and *B.sub*), in the long run, chronic inactivation of *pickle* results in loss of immune tolerance and shortened lifespan. Pickle, therefore, allows for a balanced immune response that protects from pathogenic microbes while permitting the establishment of beneficial commensal host-microbe relationships. At present little is known how the host tolerates commensal bacteria while mounting a full response to others. Our observations are consistent with a model in which Pickle acts as an immune modulator that balances the complex relationship between host resistance to pathogens and immune tolerance to microbiota. Because breakdown of this balance contributes to the development of immune-related pathologies (Pasparakis, 2009), further dissection of Pickle's unique regulatory action may aid our understanding of how aberrant NF- κ B activity contributes to dysfunction of the immune system.

EXPERIMENTAL PROCEDURES

Fly Stocks, Husbandry, and Bacterial Cultures

Flies were kept at 25°C, unless stated otherwise. A full list of all genotypes used for each figure can be found in Table S2. Bacterial cultures were initiated from single colonies grown on LB plates. Small volumes of the starter cultures were then diluted at least 1:1,000 (so as to have an near undetectable optical density [OD]) and cultured up to the desired OD on the day of the experiment.

For hk *M.lut* and *E.coli* solutions, bacteria were suspended in sterile PBS and subsequently hk for 10 min at 95°C in a heating block. Heat-killed bacterial solutions were diluted so as to enable the injection of approximately equal numbers of *E.coli* (hk) and *M.lut* (hk). Preparations were then aliquoted and frozen at -80°C for repeat use of identical hk bacterial preparations. See Supplemental Experimental Procedures for details.

Systemic Infection Experiments and Survival

Three- to eight-day-old adult flies were used for infection experiments. Systemic infection was performed by injecting flies with 13.8 nl of a cultured bacterial solution, PBS, or hk bacteria resuspended in PBS, using the Nanoject II (Drummond Scientific). Flies were then incubated at 25°C, transferred to fresh vials every day, and collected and examined at different time points for qRT-PCR, CFU counts, and survival analysis.

Oral Infection and Bleomycin Treatments and Generation of Axenic Flies

Oral infections and treatments were performed as previously reported (Buchon et al., 2009a), with some modifications. Briefly, 5- to 7-day-old female flies were raised, starved, and fed on a Whatman filter paper covered by 150 μ l of an infection solution (*Ecc15* at OD 100 or *P.e* at OD 50) or 250 μ g/ml bleomycin solution (Sigma) containing 2.5% sucrose. See Supplemental Experimental Procedures for details.

Generation of Axenic Flies

Freshly laid eggs (≤ 5 hr old) were collected from grape juice agar plates. Embryos were rinsed in 1 \times PBS, and any hatched larvae or loose agar pieces were removed with sterile forceps. All subsequent steps were performed in a sterilized laminar flow hood. Embryos were surface-sterilized by 70% ethanol and then by 5% sodium hypochlorite for 10 min, followed by three washes with sterile water, and then aseptically transferred to sterile food in a small amount of 100% ethanol. Adult female flies (about 7 days old) were collected for midgut dissection.

Lifespan Analysis

Five virgins 5966:GS homozygotes were crossed to one male with the indicated genotypes. Ten crosses were set up per genotype. Progenies were collected and allowed to mate for 2 days. Male siblings were then separated (20 flies per vial). Flies were treated with RU486, as previously described (Guo et al., 2014), with some modifications. See Supplemental Experimental Procedures for details.

Bacterial Load

The bacterial load was established as previously described (Khalil et al., 2015). Fly homogenates were serially diluted (10-fold), and CFUs were counted manually. Ten flies were analyzed per genotype and experimental repeat. A "mock" procedure lacking injected bacteria was performed in each experiment repeat. No CFUs were detectable following this "mock" procedure.

qRT-PCR and Primer Sequences

qRT-PCR was performed as previously described (Meinander et al., 2012), with some modifications. For whole-fly analysis in Figures 3, 4, and S3, pools of 15 male and 15 female flies per sample were analyzed. For whole-fly analysis in Figures 6, 7, S5, and S6, pools of 5 female flies per sample were analyzed. For midgut analysis, pools of 15–20 dissected female midguts were analyzed. The amount of mRNA detected was normalized to control *rp49* mRNA values. In Figures 5, 6, S4, and S5, the $\Delta C_t^{\text{sample}} / \Delta C_t^{\text{rp49}}$ ratios are indicated to allow comparison of the actual expression levels. For the remaining figures, relative $\Delta C_t^{\text{sample}} / \Delta C_t^{\text{rp49}}$ ratios of WT controls were set at 100%, and the fold differences were calculated using the $\Delta\Delta C_t$ method. See Supplemental Experimental Procedures for additional details and primer sequences.

Tissue Culture and Treatments

Drosophila S2* cells were a kind gift from Neal Silverman. S2* cells were cultured at 23°C in Schneider's *Drosophila* medium (Gibco), supplemented with 10% fetal bovine serum, 60 μ g/ml penicillin, and 100 μ g/ml streptomycin. RNAi knockdown was performed as described previously (<http://www.flyrnai>).

org/DRSC-PRR.html). Transfections were performed using Effectine (Qiagen) or calcium phosphate protocol (Clontech) according to the manufacturer's instructions. See [Supplemental Experimental Procedures](#) for details.

Immunoprecipitation, Nuclear and Cytoplasmic Fractionation, and Western Blot Analysis

Immunoprecipitation and western blot analysis were performed as previously described ([Meinander et al., 2012](#)), with some modifications. Cytoplasmic and nuclear fractions were separated via combined use of centrifugation and cytoplasmic and nuclear extraction buffers. See [Supplemental Experimental Procedures](#) for details.

Sequence Collection, Phylogenetic Analysis, and Model Constructions

Analysis was performed as previously described ([Basith et al., 2013](#)). The 3D model of Pickle was built using ANK-N5C (Protein Data Bank [PDB]: 4O60) as template, which shares a sequence identity of 26.8%. See [Supplemental Experimental Procedures](#) for details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.chom.2016.08.003>.

AUTHOR CONTRIBUTIONS

O.M. designed and performed the experiments. X.L. and N.B. conducted and supervised the experiments involving *Drosophila* midgut. C.D. provided advice throughout systemic infection experiments. C.R. performed preliminary experiments investigating the immune function of *pickle*. T.T. gave advice throughout western blotting experiments. A.C. and G.P. performed preliminary experiments investigating the pathological impact of *pickle*. H.C. performed preliminary experiments involving *Drosophila* midgut. S.B. and S.C. performed bioinformatics analysis. O.M. and P.M. designed the study and wrote the manuscript.

ACKNOWLEDGMENTS

We are indebted to T. Ip, L. Pile, B. Lemaitre, N. Tapon, K. Basler, M. Dionne, F. Leulier, N. Silverman, and M. Miura for fly stocks and reagents. G.P. and A.C. acknowledge assistance from the CALM Live Imaging Facility QMRI, University of Edinburgh. O.M. is supported by a Cancer Research UK studentship. S.C. is supported by the National Research Foundation of Korea (NRF-2015R1A2A2A09001059). C.D. is funded by the Biotechnology and Biological Sciences Research Council. N.B. and X.L. acknowledge funding from the Empire State Stem Cell Fund through New York State Department of Health NYSTEM contract C029542. G.P. is supported by the Wellcome Trust (Pennetta8920) and Motor Neuron Disease Association (Pennetta6231). P.M. is funded by Breast Cancer Now (2013MayPR023). We acknowledge National Health Service funding to the National Institute for Health Research Biomedical Research Centre.

Received: March 10, 2016

Revised: July 17, 2016

Accepted: August 12, 2016

Published: September 14, 2016

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Supplemental Information

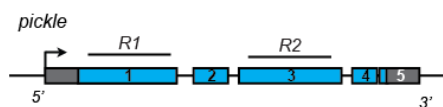
Signal Integration by the I κ B Protein Pickle

Shapes *Drosophila* Innate Host Defense

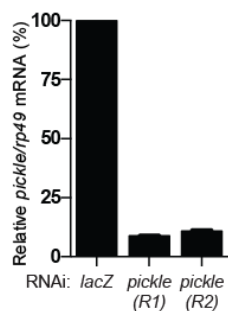
Otto Morris, Xi Liu, Celia Domingues, Christopher Runchel, Andrea Chai, Shaherin Basith, Tencho Tenev, Haiyang Chen, Sangdun Choi, Giuseppa Pennetta, Nicolas Buchon, and Pascal Meier

Supplementary Figures and Legends

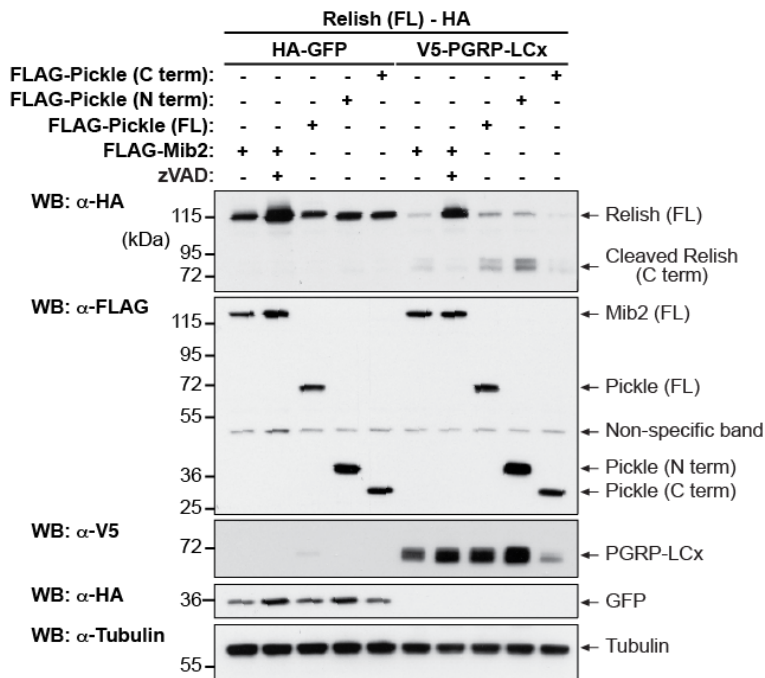
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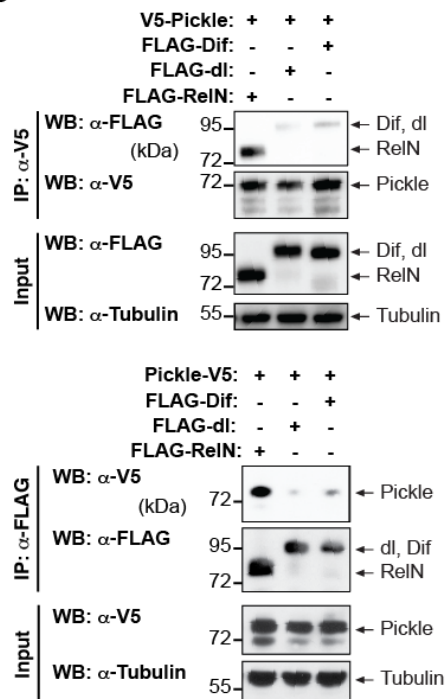
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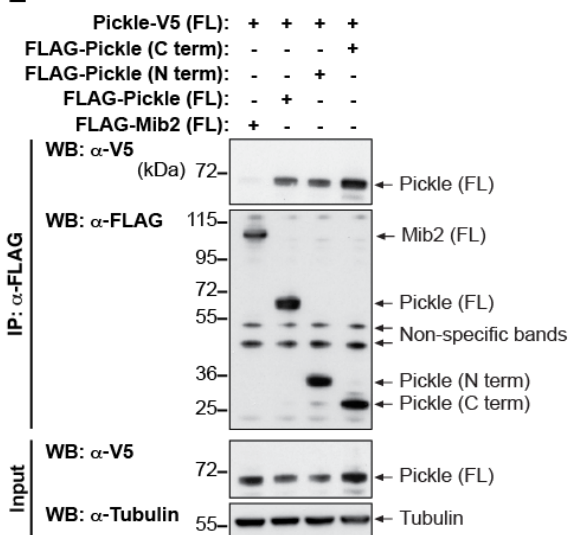


Figure S1, related to Figure 1. Pickle negatively regulates Relish

(A) Schematic representation depicting the regions that are being targeted by *pickle*'s *R1* and *R2* dsRNAs. **(B)** RT-qPCR analysis of relative *pickle* mRNA levels from S2* cells incubated with the indicated dsRNAs. **(C)** Co-expression of Pickle does not alter Relish cleavage following activation of Imd signalling by PGRP-LCx. The indicated proteins were co-expressed in S2* cells and Relish cleavage assessed via western blot. Treatment with the caspase inhibitor zVAD-FMK served as control. **(D)** Pickle selectively binds to RelN and does not interact with dl and Dif. The indicated FLAG- and V5-tagged proteins were co-expressed in S2* cells. Reciprocal FLAG and V5 immunoprecipitations were performed and binding assessed via western blot. **(E)** Pickle homo-oligomerizes. V5-tagged Pickle (FL) was expressed in S2* cells along with the indicated FLAG-tagged proteins. FLAG immuno-precipitation was performed, and binding assessed via western blot.

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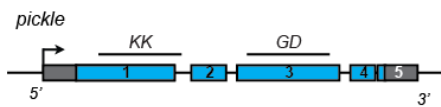
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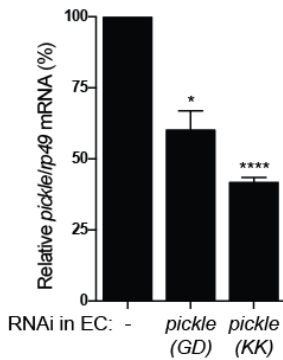
Figure S2, related to Figure 2. Phylogenetic relationship of Pickle with other IκB family members

Sequence comparisons of IκB ankyrin repeat regions (ARRs). The multiple sequence alignment of representative IκB members is shown. The amino acid numbers shown correspond to the ARR of the indicated IκB proteins. The highly conserved regions in the sequence alignment of IκB ARRs are represented in red blocks. The secondary structure prediction in relation to the Bcl-3 structure is shown at the top of the sequence alignment.

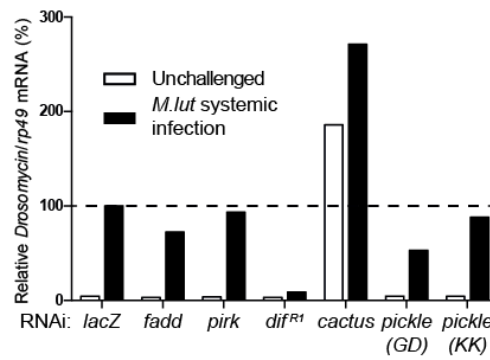
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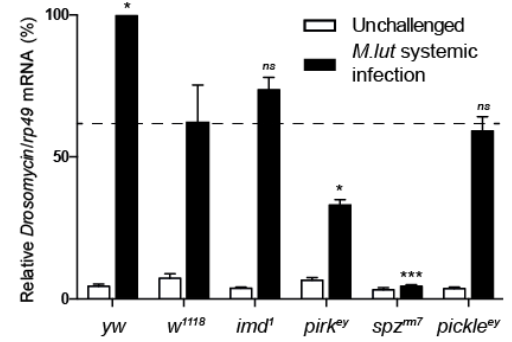
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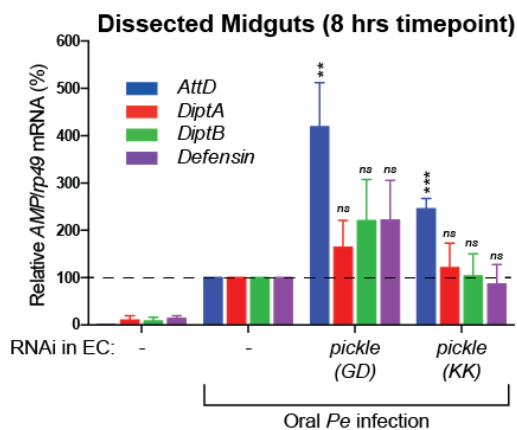
C Whole Flies (24 hr timepoint)



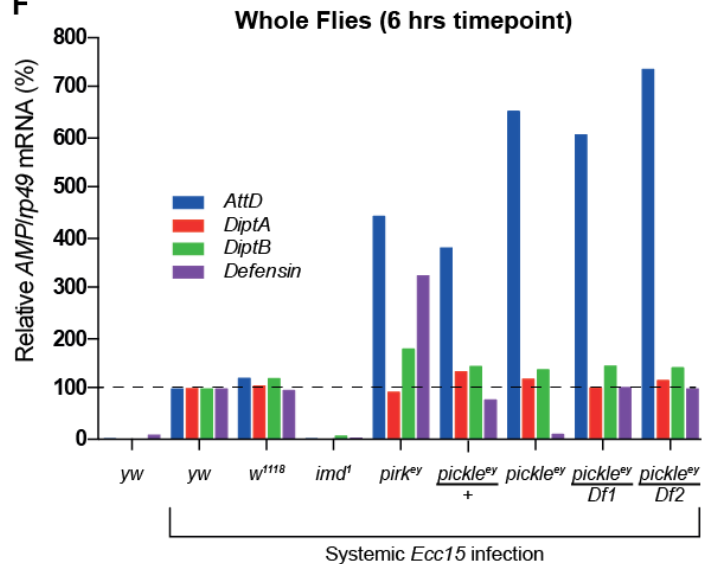
D Whole Flies (24 hr timepoint)



E



F



G

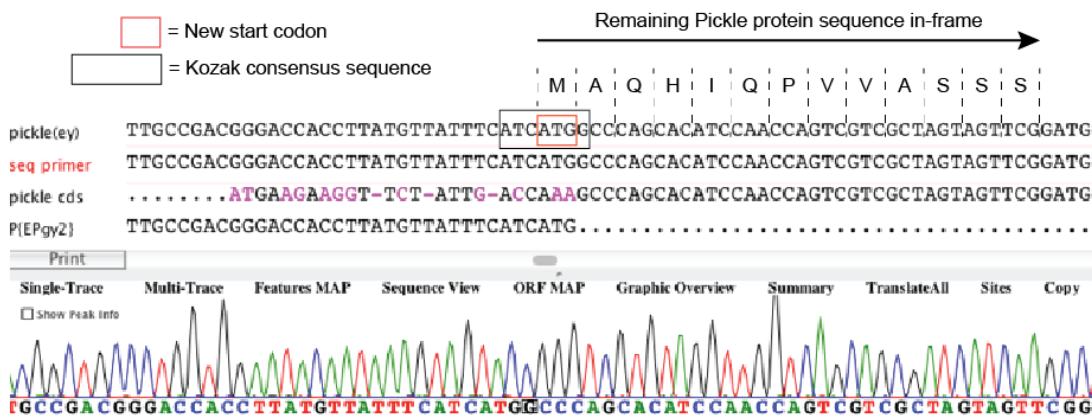


Figure S3, related to Figure 3. Loss of *pickle* causes hyper-induction of AMPs following infection with Gram-negative bacteria

(A) Scheme representation depicting the dsRNA regions that are targeted in the *pickle* RNAi lines (*GD*) and (*KK*). **(B-F)** RT-qPCR analysis of the indicated flies. (B) Relative *pickle* mRNA levels from dissected midguts. RNAi knockdown was restricted to enterocytes (EC) using *myo::Gal4*. (C, D) Relative *Drosomycin* mRNA levels from the indicated flies before and after 24 hrs of infection with *M.lut* (~200 CFU). (C) RNAi of the indicated target genes was driven in the fat body (FB) using *c564::Gal4*. (E) AMP mRNA levels of dissected midguts before and after 8 hrs of oral infection with *P.e*. RNAi knockdown was restricted to enterocytes (EC) using *myo::Gal4*. (F) Relative AMP mRNA levels from whole flies before and after 6 hrs of infection with *Ecc15* (~2000 CFU). **(G)** Sequence analysis of the *pickle^{ey}* allele depicting the position of the ATG at end of *P[EPgy2]*. Note the ATG at the end of the UAS promoter is in-frame with the remaining *pickle* coding sequence. Results were expressed as % of a control sample (marked with dotted line). Unless otherwise indicated, P values are measured from control these via an unpaired, Student *t*-test. Histograms depict mean \pm SEM of three (B, D, E) or two (C, F) biological repetitions. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$.

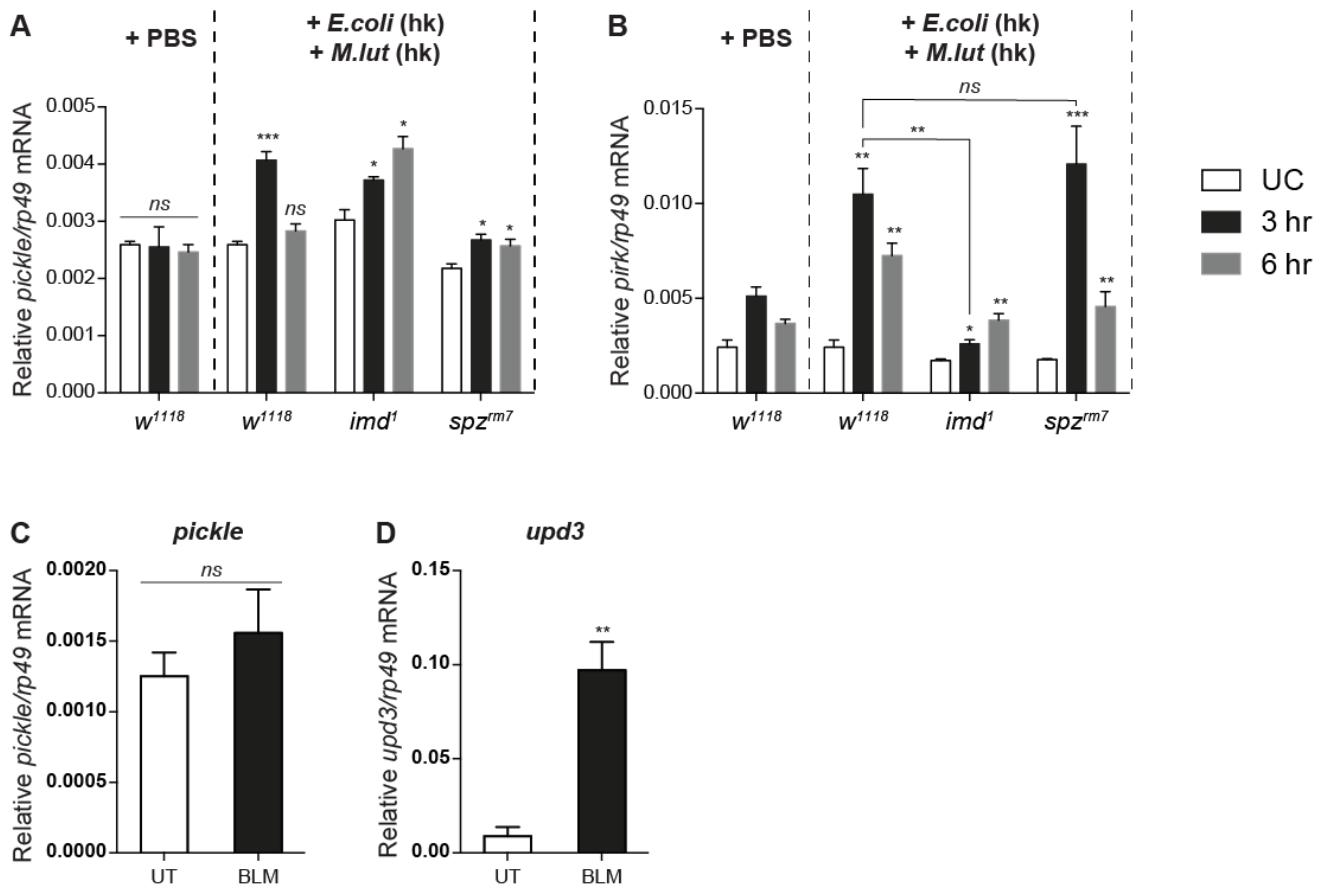
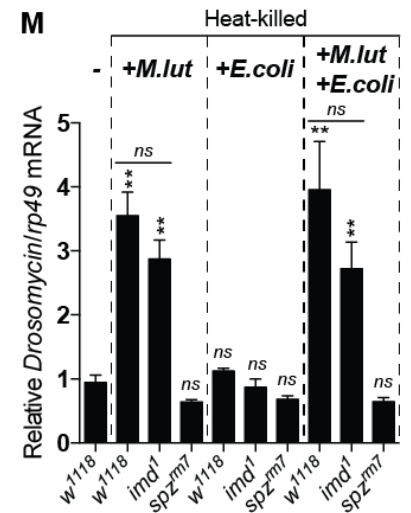
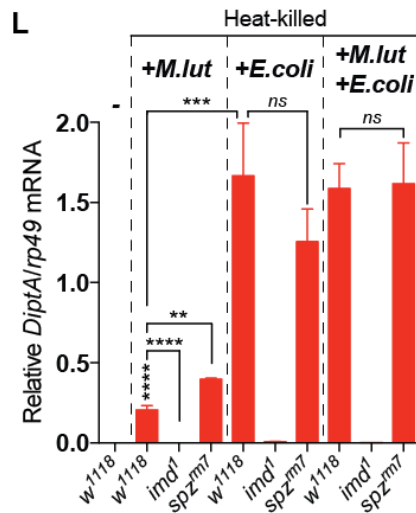
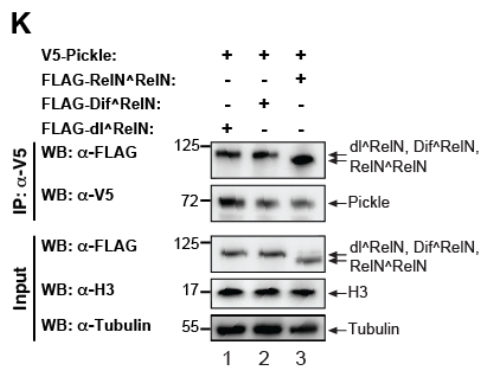
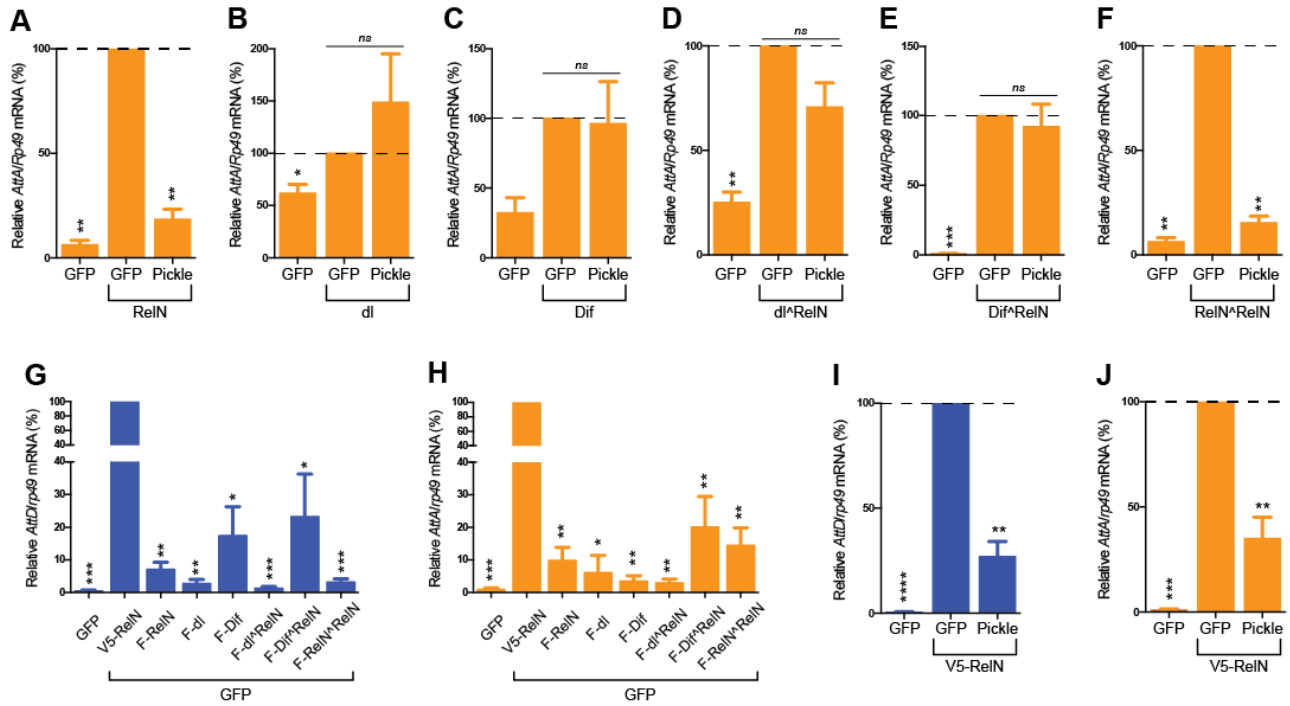
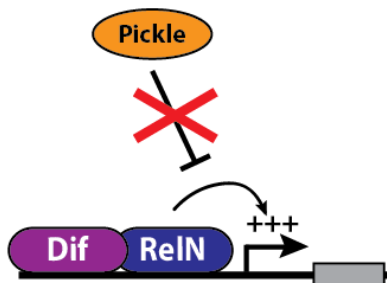


Figure S4, related to Figure 5. *pickle* expression is induced in response to commensal and infectious bacteria

(A-D) RT-qPCR analysis of *pickle*, *pirk* or *upd3* transcript levels. Relative (A) *pickle* and (B) *pirk* mRNA levels from whole flies unchallenged (UC) or injected with PBS or a heat-killed *E.coli* + *M.lut* mixture. **(C, D)** Unlike the stress-response cytokine *upd3*, *pickle* was not transcriptionally induced by damage. Relative (C) *pickle* and (D) *upd3* mRNA levels from dissected midguts of Canton S flies before and after (24 hrs) treatment with the tissue damaging agent bleomycin (BLM). Histograms depict mean \pm SEM of three biological repeats, and statistical significance was calculated using an unpaired, student *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$.



N Pickle cannot inhibit Dif:RelN heterodimers



Pickle cannot inhibit RelN:RelN homodimers when Dif:Dif homodimers are bound at the same promoter

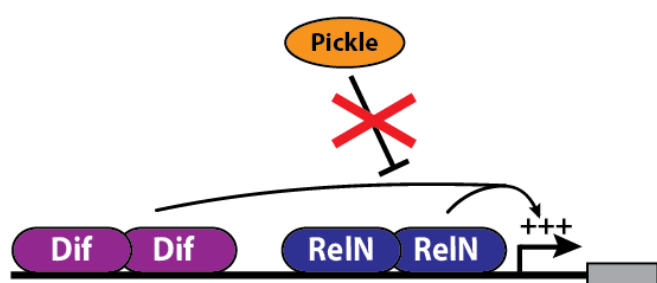
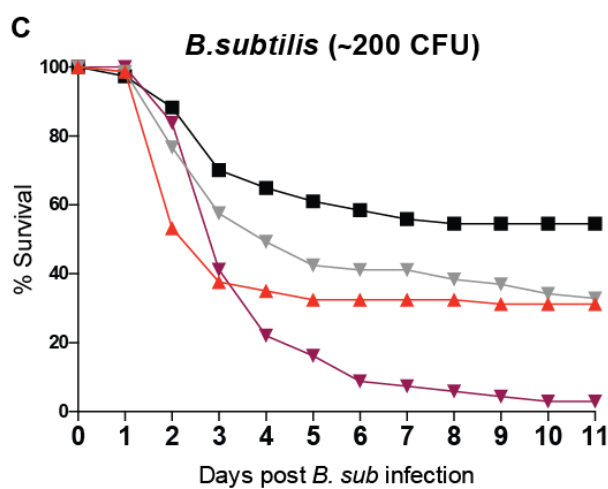
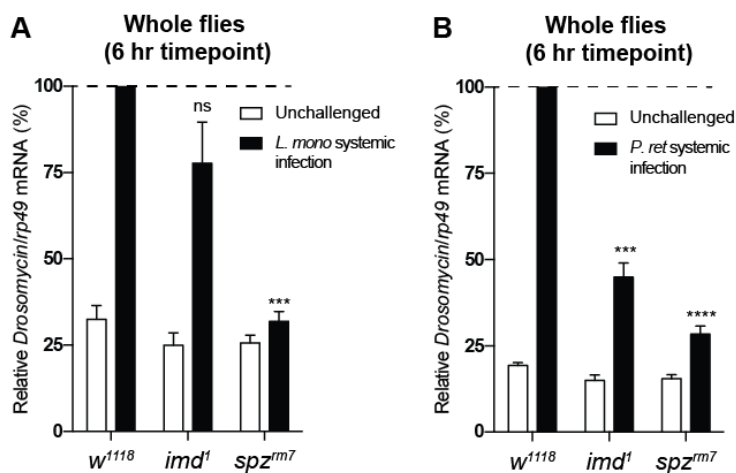


Figure S5, related to Figure 6. Pickle selectively inhibits RelN homodimers

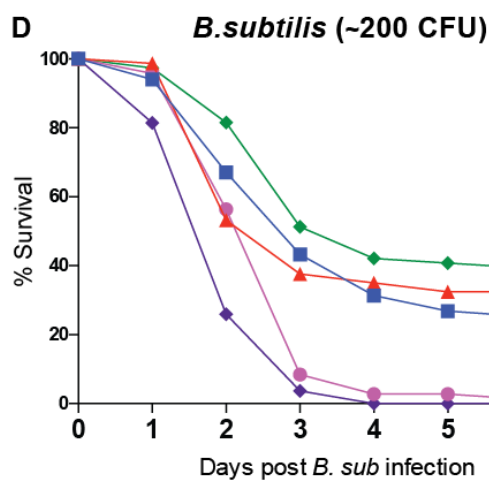
(A-F) Relative *AttA* mRNA levels of S2* cells transiently transfected with the indicated constructs. All proteins are FLAG(F)-tagged at their N-termini. Histograms depict mean \pm SEM of three biological repeats. Results are expressed as % of induced GFP control samples of each experiment, and statistical significance was measured from these via an unpaired, two-tailed Student *t*-test. **(G-J)** Relative *AttD* and *AttA* mRNA levels of S2* cells transiently transfected with the indicated constructs. Results are expressed as % of the V5-RelN + GFP sample in each experiment, and statistical significance was measured from this via an unpaired, Student *t*-test. **(K)** Pickle binds to dI^ΔRelN, Dif^ΔRelN and RelN^ΔRelN. The indicated proteins were co-expressed in S2* cells. V5 immunoprecipitation was performed and binding was assessed via western blot. **(L, M)** Relative (L) *DiptA* or (M) *Drosomycin* levels from unchallenged flies or flies injected with heat-killed *E.coli* and/or heat-killed *M.lut* (6 hrs). Unless otherwise indicated, statistical significance is measured from unchallenged *w*¹¹¹⁸ flies via an unpaired, Student *t*-test. **(N)** Depicted are two possible models that help to explain Pickle's ability to selectively inhibit RelN only when it acts alone, and not when it operates in concert with Dif.

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$.

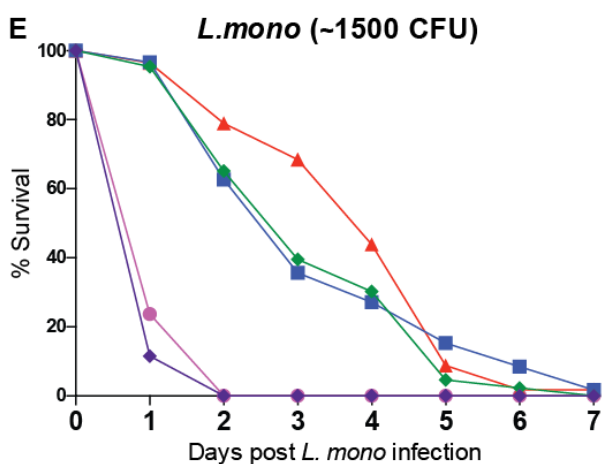


Legend	Genotype	p value (log rank test)
■	<i>pickle^{ey/DF1}</i>	0.0004
▼	<i>pickle^{ey/+}</i>	ns
▲	<i>w¹¹¹⁸</i>	-
▼	<i>pickle^{ey/c564-G4}</i>	0.0645

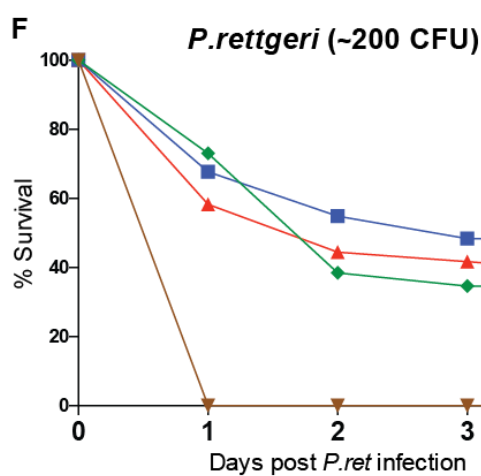
$p = 0.0085$
 $p < 0.0001$



Legend	Genotype	p value (log rank test)
▲	<i>w¹¹¹⁸</i>	-
◆	<i>pirk^{ey}</i>	ns
■	<i>yw</i>	ns
●	<i>spz^{mm7}</i>	<0.0001
◆	<i>rel²⁰</i>	<0.0001



Legend	Genotype	p value (log rank test)
▲	<i>w¹¹¹⁸</i>	-
◆	<i>pirk^{ey}</i>	ns
■	<i>yw</i>	ns
●	<i>spz^{mm7}</i>	<0.0001
◆	<i>rel²⁰</i>	<0.0001



Legend	Genotype	p value (log rank test)
▲	<i>w¹¹¹⁸</i>	-
◆	<i>pirk^{ey}</i>	ns
■	<i>yw</i>	ns
▼	<i>imd¹</i>	< 0.0001

Figure S6, related to Figure 7. Loss of *pickle* improves host resistance to pathogenic bacteria

(A, B) RT-qPCR analysis of *Drosomycin* mRNAs of samples from the indicated flies, before and 6 hrs post-systemic infection with (A) *L. monocytogenes* (~1500 CFU) and (B) *P.rettgeri* (~10000 CFU). Histograms depict mean \pm SEM of three biological repeats. Results are expressed as % of the induced levels of control flies (*w¹¹¹⁸*) in each experiment (marked with a dotted line), and statistical significance was measured from these via unpaired, two-tailed Student's *t*-test. **(C-F)** Kaplan-Meier plot showing the survival of female flies injected with (C, D) *B. subtilis* (~200 CFU), (E) *L. monocytogenes* (~1500 CFU) and (F) *P.rettgeri* (~200 CFU). Statistical significance between infected flies and a control *w¹¹¹⁸* strain were determined by log-rank tests. $n \geq 45$ flies for each genotype except *rel^{e20}* and *imd¹* genotypes, for which $n \geq 25$ flies. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$.

Supplementary Table S1, related to Figure 2. Table listing taxa terminologies of vertebrate and invertebrate I κ B proteins used in this study

Table shows name of species, species abbreviation and respective I κ B gene sequence identifiers.

Supplementary Table S2, related to Experimental Procedures. Table listing fly genotypes used in this study

Supplemental Experimental Procedures

Fly stocks, husbandry and bacterial cultures

Canton S, *w¹¹¹⁸* and *yw* flies were used as control flies as appropriate. *UAS-lacZ* flies were a kind gift from M. Miura (University of Tokyo). *UAS-pirk-IR* and *pirk^{ey}* flies were described previously (Lhocine et al., 2008). *UAS-fadd-IR* (VDRC 100333), *UAS-pickle(GD)-IR* (VDRC 34936) and *UAS-pickle(KK)-IR* flies (VDRC 106292) were obtained from VDRC stock centre. Note that VDRC 34936 is no longer publically available at VDRC. *Df(2L)Exel7006*, *Df(2L)BSC481*, *pickle^{ey}*, *UAS-relish-IR* (TRIP 33661), *UAS-cactus-IR* (TRIP 34775), *UAS-dif(R1)-IR* (TRIP 30513), *UAS-dif(R2)-IR* (TRIP 29514), *UAS-dl(R1)-IR* (TRIP 27650) and *UAS-dl(R2)-IR* (TRIP 34938) were obtained from Bloomington stock centre. *UAS-pickle* flies were obtained from NCBS *Drosophila* Resource Centre. *imd¹*, *spz^{rm7}*, *rel^{e20}*, *PGRP-LC^{E12}* and *PGRP-LE¹¹²* flies were described previously (Neyen et al., 2014). Flies were maintained at 25°C and 65% humidity on a 12 hrs light/dark cycle, unless otherwise indicated. A full list of all genotypes used for each Figure can be found in Supplemental Table S2.

With the exception of *L.monocytogenes*, starter cultures of all bacterial cultures were initiated from single colonies grown on LB plates. Small volumes of the starter cultures were then diluted at least 1:1000 (so as to have an near undetectable OD) and cultured up to the desired OD on the day of the experiment. *Ecc15* was cultured in LB at 29°C with agitation to OD 0.3. *M.luteus* was cultured in LB at 37°C with agitation to OD 0.3. *P.ret* was cultured in LB at 37°C with agitation to OD 0.1 (low dose) or OD 1 (high dose). *B.subtilis* was cultured in LB at 37°C with agitation to OD 0.1. As an exception, *L.mono* was cultured overnight without agitation at 37°C in brain heart infusion (BHI) broth. The culture was initiated from a single colony grown on a BHI plate. The resulting overnight culture was then serially diluted to OD 0.2 using BHI broth.

Heat-killed (hk) bacteria were prepared as follows: *E.coli* (1106 strain) and *M.luteus* were cultured overnight in LB at 37°C with agitation. Cultures were initiated from single colonies grown on LB plates. After 24 hrs, cultures were spun down at 1600 rpm for 4 minutes at 4°C. Culture media was aspirated and remaining bacterial pellets were resuspended with sterile PBS to OD 1. Bacterial solutions were then heat-killed (hk) for 10 minutes at 95°C in a heating block. Heat-killed bacterial solutions were then prepared as followed: hk *M.lut*-only preparation = 9 volumes OD 1 *M.lut* (hk) suspension + 1 volume sterile PBS. *E.coli* (hk) only preparation = 1 volume OD 1 *E.coli* (hk) suspension + 9 volume sterile PBS. Mixtures of *M.lut* (hk) + *E.coli* (hk) preparation = 9 volumes OD 1 *M.lut* (hk) suspension + 1 volumes OD 1 *E.coli* (hk) suspension. These dilutions enabled the injection of approximately equal number of *E.coli* (hk) and *M.lut* (hk) respectively (live *E.coli* OD 1 contains approximately ten times as many CFUs as live *M.lut* OD 1). Preparations were then aliquoted and frozen at -80°C for repeat usage of identical hk bacterial preparations.

Oral infection and bleomycin treatment.

5-7 days old female flies were collected and kept at 18°C for 3 days (Gal80^{TS} ON, Gal4 OFF), and then were incubated at 29°C for seven days to knock-down the target gene (Gal80^{TS} OFF, Gal4 ON, RNAi ON). Flies were starved for 2 hrs at 29 °C and then transferred to infection vials covered with a filter paper (WhatmanTM) and 150 µl of an infection solution (*Ecc15* at OD 100, or *P.e.* at OD 50 in 2.5 % sucrose). Flies were dissected as described previously (Buchon et al., 2009; Houtz and Buchon, 2014). For bleomycin treatment, 5-7 day old female flies were raised, starved and fed on a Whatman filter paper covered by 150 µl of a solution of 250 µg/ml bleomycin (Sigma) in 2.5 % sucrose.

Lifespan analysis.

5 virgins 5966::GS homozygotes were crossed to 1 male homozygote *UAS-lacZ-IR*, *UAS-pickle(GD)-IR* or *UAS-pickle(KK)-IR* in fly vials. 10 crosses were set per genotype. Progeny of these crosses were collected for 5 days after the first fly hatched. Flies were then allowed to mate for 2 days. Male siblings were then separated (20 per vial). For RU486 food supplementation, 100 µl of a 5 mg/ml solution of RU486 or vehicle (ethanol 80%) was deposited on top of the food and dried for at least 16 hrs. This resulted in a 0.2 mg/ml concentration of RU486 in the food accessible to flies (determined using a dye control as previously described for drug treatments (Grover et al., 2009)).

RT-qPCR and primer sequences.

RNA was extracted from whole flies, dissected midguts or S2* cells using either Trizol, Qiazol, Direct-zol RNA Mini Kit (Zymo Research) or RNeasy Mini Kit (QIAGEN). For whole fly analysis in Figures 3, 4, S3; pools of 15 male + 15 female flies were analysed. For whole fly analysis in Figure 6, 7, S5 and S6; pools of 5 female flies per sample were analysed. cDNAs were synthesized using Quantitect Reverse transcription kit (QIAGEN), and quantitative PCR was performed using MESA Blue qPCR mastermix Plus for SYBR assays (Eurogentech). SYBR green analysis was performed on a 7900HT machine (Applied Biosystems). For midgut analysis, a pool of 15 to 20 dissected female midguts was collected into Trizol (Invitrogen) for RNA extraction. Reverse transcription (RT) reactions were performed using the qScript cDNA synthesis kit (Quanta). Quantitative PCR was performed with SsoAdvancedTM Universal SYBR® Green Supermix (Bio-Rad) using CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad).

RT-qPCR SYBR Primer list.			
CG number	Name	Forward	Reverse
CG7939	<i>rp49</i>	GACGCTTCAAGGGACAGTATCTG	AAACGCGGTTCTGCATGAG
CG7629	<i>Attacin D</i>	GTCAGTAGGGTTCCTCAG	GCCGAAATCGGACTTG
CG12763	<i>Diptericin A</i>	GCTGCGCAATCGCTTCTACT	TGGTGGAGTGGGCTTCATG
CG10794	<i>Diptericin B</i>	AGCCTGAACCACTGGCATA	AGATCGAATCCTTGCTTTGG
CG1385	<i>Defensin</i>	GTTCTTCGTTCTCGTGG	CTTTGAACCCCTTGGC
CG10810	<i>Drosomycin</i>	CGTGAGAACCTTTTCCAATATGATG	TCCCAGGACCACCAGCAT
CG5118	<i>pickle</i>	AACGGAAGAAAAGGGATGTACC	GTTTCGGCGTACAGGGGTTTTA
CG15678	<i>pirk</i>	GGCGTTCGTGTGATAG	CTCAATGCGGTACTCC
CG33542	<i>unpaired 3</i>	GCGGGGAGGATGTACC	GTCTTCATGGAATGAGCC

Tissue culture and treatments.

Drosophila S2* cells were a kind gift from Neal Silverman. S2* cells were cultured at 23°C in Schneider's *Drosophila* medium (Gibco) supplemented with 10% FBS, 60 µg/ml Penicillin and 100 µg/ml Streptomycin. For transfection experiments, cells were seeded at 1×10^6 cells/ml density 1 day prior to transfections. For IPs/western blots, calcium phosphate (Clontech, Palo Alto, CA) was used to transiently transfect 3 µg total plasmid DNA (1:2 bait to prey ratio for IPs) according to manufacturer instructions. For RT-qPCR experiments, Effectene (QIAGEN) was used to transiently transfect 0.4 µg total plasmid DNA (1:4 inducer to *gfp/pirk/pickle* ratio) according to manufacturer instructions. For RT-qPCR experiments only, 1 day following transfection, cells were split back to 1×10^6 cells/ml density. Copper sulphate-inducible pMT vectors (ThermoFisher) were used throughout. Cells were treated with 0.7 mM copper sulphate two days after transfection, and collected after 6 hrs. RNAi knockdown was performed using 20 µg dsRNA as described previously (<http://www.flyrnai.org/DRSC-PRR.html>). For RNAi experiments, cells were subsequently treated with 1 µg/ml DAP-PGN (isolated from *E.coli*) and collected after 4 hrs. In all tissue culture experiments, cells were treated with 1 µM 20-hydroxyecdysone (Sigma) for 24 hrs prior to collection.

Immunoprecipitation, nuclear/cytoplasmic fractionation and western blot analysis.

For immunoprecipitation, cells were lysed on ice in S(+M) lysis buffer containing: 20 mM Tris pH 8.0, 40 mM Na₂P₂O₇, 50 mM NaF, 5 mM MgCl₂, 100 µM Na₃VO₄, 10 mM EDTA, 1 % Triton X-100, 0.5 % NaDOC plus Halt protease + phosphatase inhibitors (ThermoFisher). Lysates were run through a column (ThermoFisher) and total cell extracts were collected. FLAG or V5-tagged proteins were purified using antibody-IgG-coupled agarose beads (Sigma Aldrich, GE Healthcare respectively). After 2 hrs incubation at 4°C, beads were washed three times in S(+M) wash buffer: 20 mM Tris pH 8.0, 40 mM Na₂P₂O₇, 50 mM NaF, 5 mM MgCl₂, 100 µM Na₃VO₄, 10 mM EDTA, 0.1 % Triton X-100. For nuclear/cytoplasmic fractionation, cells were lysed on ice in cytoplasmic extraction buffer containing: 50 mM Tris pH 7.5, 137 mM NaCl, 10 % glycerol, 0.5 % Triton X-100. Extracts were spun at 14,000 r.p.m for 15 minutes and supernatants collected (cytoplasmic fraction). The remaining nuclear pellet

was washed three times in cytoplasmic extraction buffer and subsequently lysed in nuclear extraction buffer: 50 mM Tris pH 7.5, 137 mM NaCl, 10 % glycerol, 0.5 % Triton X-100, 1.5% SDS. The nuclear extract was vortexed, boiled and purified through a column (ThermoFisher) to ensure complete lysis. The following antibodies were used for western blotting: α -FLAG M2 (Sigma), α -V5 (Serotec), α -HA (Roche), α -Relish (ABIN1111036, antibodies-online), α -dHDAC1 (gift from Lori Pile), α -Tubulin (T9026, Sigma) and α -Histone 3 (MBL). Signals were visualised via chemiluminescence (GE Healthcare or Biorad Clarity).

Sequence collection and phylogenetic analysis

We used 11 biochemically characterized I κ B protein sequences from *Homo sapiens* and *Drosophila melanogaster* sources (I κ B α _Hs-CAB65556, I κ B β _Hs-AAH15528, I κ B ϵ _Hs-NP_004547, Bcl3_Hs-NP_005169, I κ BNS_Hs-Q8NI38, I κ B ζ _Hs-Q9BYH8, NF- κ B1_Hs-AAA36361, NF- κ B2_Hs-CAC08399, Cactus_Dme-AAA85908, Relish_Dme-AAB17264 and Pickle_Dme-AAF51401.2) as query sequences for constructing I κ B dataset. We constructed the dataset by utilizing the same sequences as mentioned in our previous analysis (Basith et al., 2013). Furthermore, few updated I κ B sequences along with Pickle and its homologs were included in our new dataset. The Pickle homolog sequences (57 initial sequences) were obtained through BLAST search. Most of the sequences were discarded based on the filtering criteria described previously. The final twelve Pickle homologs were included in our final dataset for phylogenetic reconstruction. Since the previous dataset was too large, we included only a maximum of five I κ B representative sequences (only in the case of vertebrates) for each species in our dataset. All I κ B sequences were imported into the Geneious Trial software v8.1.2 (Kearse et al., 2012). Multiple sequence alignment (MSA) was performed using the plugin MAFFT v7.017 (Katoh et al., 2002) implemented in Geneious software. The MSAs were manually inspected and a few particularly gap-rich positions, poorly aligned and divergent regions from the alignments were excluded prior to the phylogenetic analysis. The final dataset contained a total of 148 sequences (including outgroup) from 75 organisms that were subjected to phylogenetic tree reconstructions (17 I κ B α , 5 I κ B β , 18 I κ B ϵ , 8 Bcl3, 7 I κ BNS, 11 I κ B ζ , 11 Cactus, 25 Relish, 17 NF- κ B1, 16 NF- κ B2, and 12 Pickle sequences). The MSAs were utilized to construct two rooted and two unrooted phylogenetic trees by UPGMA and neighbor joining (NJ) methods in Geneious Trial software v8.1.2. The molecular distances between the aligned sequences were calculated using the Jukes-Cantor genetic distance model. All gaps and missing data in the alignments were accounted for by pairwise deletions. Branch points were tested for significance by bootstrapping with 1000 replications and the most probable consensus tree was calculated with a support threshold of 50% for whole I κ B phylogeny (combined vertebrate and invertebrate phylogeny) and 60% for invertebrate phylogeny constructions.

Model construction.

The three-dimensional (3D) model of Pickle was built using ANK-N5C (PDB ID: 4O60) as template, which shares a sequence identity of 26.8%. The target-template sequence alignments were

performed using MUSCLE (Edgar, 2004). The model was built using Modeller 9v8 (Eswar et al., 2006). We constructed 3D models using a distance restraint algorithm based on the MSA of the target sequence with the template structure by applying the CHARMM force field (Jo et al., 2008). An optimization method, which involved conjugate gradients and MD-simulated annealing, was employed to minimize violations of spatial restraints. For model building, the default parameters included in the “automodel” class were used. A series of 20 models were built, from which the best final model was selected based on RFMQA score. The quality of the models was assessed using RFMQA (Manavalan et al., 2014).

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